Microbiology of membrane bioreactors for wastewater treatment: a molecular approach

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Abstract

Water is a natural resource essential for life maintenance of the human kind and the ecosystems found in Nature. Currently the natural resources of water are increasingly exhausted with the constant water demand. Biological wastewater treatment processes are green, economic and efficient ways to remove pollutants from wastewater and recycle the water back into the environment. Membrane bioreactors (MBRs) in the last two decades have gained special attention due to the small footprint and the high quality of the treated effluent, helping meeting the requirements of increasingly stricter legislations. MBRs combine the activated sludge process with membrane filtration and have specific features that likely affect the microbial structure and ecophysiology.

The work presented in this thesis had as an overall objective obtaining a deep and fundamental knowledge about the microbiology underlying the MBRs' performance, which can in the future support the improvement of this technology. One of the aims in this thesis was to elucidate the potential of the MBR in promoting a better nutrient removal of the wastewater by the proliferation of important functional microorganism groups. Another aim concerning the microbial population was to assess the influence of typical MBR operational conditions on the community structure, as well as on the extracellular polymeric substances (EPS) profile, and understand the implications of these two microbiological factors in MBR performance.

MBRs can lead to significant improvements in biological nutrient removal from wastewater, due to the full retention of the biomass and the possibility to employ long sludge retention times (SRT) that promote the proliferation of slow-growing organisms. This study includes a comprehensive analysis of the

microorganisms involved in phosphorus removal from the wastewater, the polyphosphate accumulating organisms (PAOs), in MBRs. Although the storage of phosphorus is typically achieved by recirculating the activated sludge between anaerobic and aerobic conditions, PAOs were present in similar levels in all studied MBRs, with an overall appreciable phosphorus removal, regardless if an anaerobic zone was present. This data suggested that PAOs encounter in MBRs not only conditions to thrive but also to take up phosphorus from the wastewater, presumably finding areas of anaerobic micro-niches within the sludge flocs in poorly mixed zones and having selective advantage in fulfilling cell maintenance requirements in such substrate-limited conditions.

The microorganisms present in MBRs thrive in an aggregative form of life, whether as suspended flocs or as biofilm attached to the membrane. The EPS form the matrix responsible for such aggregation and can be divided into soluble and bound EPS. In this study the microbial community developed in MBRs was characterized as well as the extracellular polymeric substances (EPS) produced by those communities. The microbial and EPS profiles, together with other process parameters, were monitored throughout several months of operation where the reactor was subjected to changes of the sludge retention time (SRT), in order to investigate the impact of this parameter on the diversity and ecophysiology of the biomass. The metagenome of the bacterial community of the mixed liquor and from the cake layer was assessed by Illumina sequencing. The protein profile of the EPS of the suspended flocs was investigated by mass spectrometry (MS). The overall bacterial community structure of the mixed liquor was moderately influenced by changing the SRT from 60 to 20 days. In the cake layer, imposing a shorter SRT lead to a shift in the population, favoring the proliferation and, ultimately, the predominance of the *Actinobacteria* class. This selection for the

Actinobacteria class had no parallel in the mixed liquor community, and under the SRT of 20 d the cake layer and the suspended flocs populations were significantly distinct. Previously to the proteomic study of this reactor, the separation and identification of the extracellular proteins was optimized, and a novel workflow was proposed for successful EPS proteome characterization. The protein profile of the soluble and bound EPS of the suspended biomass flocs was composed by a high diversity of bacterial protein species, associated to different biological processes and molecular functions. Soluble and bound EPS had distinct protein profiles, and some proteins were exclusive to the soluble fraction. SRT was partially correlated with the appearance of a group of 14 proteins, detected mainly in samples taken during the 60 d SRT period. The majority of the identified proteins are involved in transport and in stress response. The stressful conditions, to which the biomass is apparently exposed, seem to affect less the community structure than protein expression. In the cake layer the stressful environment also impacted in the diversity of the community, with some bacterial groups showing a better adaption capacity to such conditions.

Fouling in MBRs occurs by the accumulation of molecules and cells at the membrane surface and pores. EPS are known to be a major contributor to fouling in MBRs. Two distinct fouling layers can form at the membrane surface, the gel layer and the cake layer, with different contributions from the soluble and bound EPS. In this study, the impact of the sludge retention time (SRT) on the composition of the EPS foulants of these two layers was investigated by operating the system at a SRT of 60 and 20 days. At the end of each SRT the cake layer was collected and the membrane modules were autopsied for gel layer analysis, where five autopsy methods were compared in terms of extraction efficiency of polysaccharides and proteins from hollow fibres located in the internal and

external parts of the membrane module. Polysaccharides were the EPS molecules comprising the gel layer. In the cake layer, the proteins had a more preponderant presence. The cake layer proteins from the end of the 20 d SRT period were identified and a significant group was stress-related, which is in agreement with the proteins identified from the EPS of the suspended flocs when subjected to the same SRT.

Overall this work contributes with a broad knowledge about the microbiology underlying the MBRs performance. The characterization of the microbial community and of the EPS produced by such populations in MBRs is a valuable tool to understand the functional potential of the system, as different bacterial populations have different functional characteristics that impact in the wastewater treatment. The know-how about the influence of the biomass on the membrane fouling is a step forward that can contribute to the improvement of the MBR operation and to its wider application.

Sumário

A água é um recurso natural fundamental à sobrevivência do Homem e dos ecossistemas. Atualmente a escassez de água não contaminada no meio ambiente leva à procura de tecnologias de tratamento de águas residuais que aumentem este recurso e respondam eficazmente às legislações aplicadas, cada vez mais rigorosas. O tratamento biológico implementado nas estações de tratamento das águas residuais é um método económico, eficiente e sustentável. Os biorreatores de membranas combinam o tratamento biológico com a filtração por membrana. Nas últimas décadas esta tecnologia atraiu um grande interesse devido às vantagens que apresenta relativamente ao tratamento convencional por lamas ativadas, nomeadamente a excelente qualidade do efluente tratado, as dimensões menores das estações de tratamento, a menor produção de lamas excedentes, e a flexibilidade da operação. Relativamente à operação de um biorreator de membrana, este apresenta características específicas, como a capacidade de ser operado a tempos de retenção de lamas longos e a concentrações de biomassa elevadas. Tais condições têm consequências na estrutura e ecofisiologia das populações microbianas.

A caracterização das comunidades microbianas presentes neste tipo de reatores biológicos é uma ferramenta valiosa para compreender o funcionamento destes sistemas, tendo em conta que populações bacterianas diferentes terão características funcionais diferentes, podendo ser relevantes ou problemáticas para o tratamento das águas residuais. O trabalho apresentado ao longo desta tese teve como objetivo geral obter um conhecimento profundo e fundamental acerca da microbiologia dos biorreatores de membranas e contribuir a longo prazo para o aperfeiçoamento desta tecnologia. Para tal, esta tese visou elucidar o

potencial dos biorreatores de membrana em promover a proliferação de populações microbianas fundamentais para a remoção de nutrientes assim como a sua ação no tratamento da água. Um outro objetivo explorado foi conhecer as populações microbianas que se desenvolvem neste tipo de reatores, sob determinadas condições de operação, e perceber as implicações destes microrganismos no desempenho do reator. Além de caracterizar as populações, foram também estudadas as substâncias poliméricas extracelulares produzidas por estas populações, conhecidas por serem agentes de *fouling* da membrana, o grande obstáculo à aplicação em massa desta tecnologia.

A retenção total da biomassa num biorreator de membranas e a operação a tempos de retenção de lamas longos pode conduzir a uma melhor remoção biológica dos nutrientes devido à proliferação de microrganismos com taxas de crescimento lento. Esta tese apresenta uma análise abrangente das populações microbianas envolvidas na remoção de fósforo das águas residuais. Tipicamente a remoção de fósforo é conseguida através da recirculação das lamas ativadas entre condições aeróbias e anaeróbias de modo a fomentar a seleção dos microrganismos que removem o fósforo. Contudo, no geral estes organismos encontraram nos biorreatores de membranas condições tanto para proliferarem como para removerem o fósforo do influente, mesmo quando um compartimento anaeróbio não estava presente. Provavelmente, os organismos acumuladores de fósforo encontraram micro nichos anaeróbios no interior dos flocos de biomassa, para além de possuírem uma vantagem seletiva ao acumularem fósforo no seu interior, uma fonte de energia a ser usada em condições limitativas de substrato, como ocorre num biorreator de membrana.

Os microrganismos proliferam nos biorreactores de membrana na forma de agregados, quer na forma de flocos em suspensão quer na forma de biofilmes à

superfície da membrana. As substâncias poliméricas extracelulares formam a matriz responsável por esta agregação dos microrganismos, que pode ser dividida numa fração solúvel e outra ligada às células. As populações microbianas assim como as substâncias poliméricas extracelulares presentes num biorreator de membranas foram estudadas nesta tese. O MBR foi sujeito a dois tempos de retenção de lamas distintos, um mais longo (de 60 dias) seguido de um mais curto (de 20 dias), para investigar o impacto das condições de operação na microbiologia e desempenho do sistema. As populações bacterianas foram identificadas pela técnica de sequenciação de nova geração Illumina, e as proteínas do metaproteoma extracelular por espetrometria de massa. A população bacteriana presente nos flocos em suspensão foi moderadamente afectada pela alteração de tempo de retenção de lamas. No bolo, a mudança de tempo de retenção de lamas para um período mais curto foi acompanhada por uma alteração drástica na população, com predominância da classe *Actinobacteria*, sendo esta população muito distinta da correspondente dos flocos em suspensão. Anteriormente ao estudo do metaproteoma extracelular, método de separação e identificação de proteínas foi otimizado, culminando com a proposta de uma estratégia passo-a-passo para uma caracterização com sucesso de proteínas que compõem as substâncias poliméricas extracelulares. A metodologia otimizada foi aplicada nos trabalhos subsequentes, apresentados nesta tese. A partir da análise das proteínas extracelulares dos flocos em suspensão, pode-se verificar que o tempo de retenção de lamas teve um impacto no perfil de um grupo de 14 proteínas, que surgem maioritariamente no período de 60 dias. Igualmente, o perfil de proteínas das substâncias poliméricas solúveis é estatisticamente distinto do perfil observado na fração ligada às células. Um grande número de proteínas está envolvido na resposta ao stress, o que leva a crer que as populações estão sujeitas a condições de stress dentro do MBR. Estas

condições parecem afetar mais a expressão proteica do que a estrutura da comunidade bacteriana, embora no bolo a resposta a estas condições se tenha refletido numa variação da composição da população bacteriana.

O *fouling* da membrana ocorre devido à deposição e adesão de células e moléculas à superfície da membrana. Formam-se, deste modo, dois tipos de camadas à superfície da membrana, a camada gel através da deposição de substâncias poliméricas em solução, e o bolo devido à compactação dos flocos de biomassa à superfície da membrana e ao crescimento do biofilme. As principais moléculas que compõem as substâncias extracelulares poliméricas, as proteínas e os polissacáridos, foram caracterizadas em cada uma destas duas camadas de *fouling*. O bolo foi recolhido e a camada gel foi extraída por autópsia da membrana. Cinco métodos de autópsia foram comparados em termos de eficiência de extração das proteínas e dos polissacáridos a partir da camada gel de fibras localizadas nas partes externa e interna do módulo de membranas. Os resultados obtidos demonstraram que os polissacáridos são as moléculas responsáveis pela formação da camada gel, enquanto que as proteínas são as moléculas preponderantes no bolo. Por espectrometria de massa, foi possível identificar as proteínas do bolo no final do período operado com retenção de lamas inferior, e verificar-se que um grupo significativo estava implicado na resposta a condições de stress.

Esta tese representa um avanço na compreensão das populações microbianas envolvidas nos processos biológicos que ocorrem num biorreator de membranas, assim como das substâncias poliméricas expressas por tais populações. Este conhecimento é fundamental e importante para aplicações futuras no melhoramento do desempenho dos reatores de membranas, tanto a nível da remoção de nutrientes como a nível do controlo do *fouling* da membrana.

Peer-reviewed Articles

Thesis publications

Silva AF, Carvalho G, Soares R, Coelho AV, Barreto Crespo MT (2012) Step-by-step strategy for protein enrichment and proteome characterization of extracellular polymeric substances in wastewater treatment systems. Appl Microbiol Biotechnol 95(3):767-76

Silva AF, Carvalho G, Oehmen A, Lousada-Ferreira M, van Nieuwenhuijzen A, Reis MA, Crespo MT (2012) Microbial population analysis of nutrient removal-related organisms in membrane bioreactors. Appl Microbiol Biotechnol 93(5):2171-80

Additional publications

Albuquerque MG, Carvalho G, Kragelund C, **Silva AF**, Barreto Crespo MT, Reis MA, Nielsen PH (2013) Link between microbial composition and carbon substrate-uptake preferences in a PHA-storing community. ISME J 7(1):1-12

Abbreviations

AOA	Archaeal Ammonia Oxidizers
AOB	Ammonia-Oxidizing Bacteria
BNR	Biological Nutrient Removal
BOD	Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
CAS	Conventional Activated Sludge
CER	Cation Exchange Resin
CID	Collision Induced Dissociation
CLSM	Confocal Laser Scanning Microscope
COD	Chemical Oxygen Demand
DAPI	4',6-Diamidino-2-Phenylindole
DGGE	Denaturating Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DOC	Deoxycolate
DPAOs	Denitrifying Polyphosphate Accumulating Organisms
EBPR	Enhanced Biological Phosphorus Removal
EDTA	Ethylenediamine Tetraacetic Acid
EPS	Extracellular Polymeric Substances
F/M	Food to Microorganisms Ratio
FISH	Fluorescence *in situ* Hybridization
FITC	Fluorescein Isothiocyanate
FS	Flat Sheet
GAOs	Glycogen Accumulating Organisms
HF	Hollow Fiber
HRT	Hydraulic Retention Time
IQR	Interquartile Range
J	Permeate Flux
MALDI-ToF/ToF	Matrix-Assisted Laser Desorption/Ionization - Tandem Time-Of-Flight
MBRs	Membrane Bioreactors
MES	2-(N-Morpholino) Ethanesulfonic Acid
MLSS	Mixed Liquor Suspended Solids
MS	Mass Spectrometry
MW	Molecular Weight
NOB	Nitrite-Oxidizing Bacteria
OTU	Operational Taxonomic Unit
PAOs	Polyphosphate Accumulating Organisms
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PHA	Poly-β-Hydroxylalkanoate

PP	Polyacrylate-Polyalcohol
Q_{aer}	Aeration Flow Rate
RNA	Ribonucleic Acid
SAD	Specific Aeration Demand
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SE	Standard Error
SRT	Sludge Retention Time
TCA	Trichloroacetic Acid
TMP	Transmembrane Pressure
t-RFLP	Restriction Fragment Length Polymorphism
UPGMA	Unweighted Pair Group Method Of Arithmetic Averages
VFA	Volatile Suspended Solids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plants

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CHAPTER 1

Introduction

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Wastewater has essentially an anthropogenic origin and is mainly a combination of human faeces, urine, greywater and microorganisms excreted by humans (Maier *et al*. 2000). It can also include industrial and hospital influents. The need to treat wastewater before its discharge to water bodies came with the threat of waterborne diseases and the concern with the negative impact in the aquatic environment in the middle of the 19th century. Only from the 20th century on, specific processes were developed to reduce the organic matter of wastewater before its discharge into the environment. With the increased demand on potable water and the decrease of pristine water sources, these processes also started to include the reduction of pathogenic microorganisms and removal of toxic substances in wastewater treatment plants (WWTP) (Seviour and Nielsen 2010).

In the WWTP, the primary goal is to reduce the organic matter to levels within wastewater discharge permits, which is normally evaluated by the biochemical/chemical oxygen demand (BOD and COD respectively). The ratio between these two parameters gives an estimate of the efficiency potential of the biological treatment process, since the BOD gives an assessment of the biodegradable organic matter, whereas the COD measures all the organic matter in the water. Further treatment focuses the removal of other nutrients, such as nitrogen and phosphorus, as well as pathogenic microorganisms and other pollutants present in lower concentrations, such as heavy metals and pharmaceuticals (Leslie Grady Jr *et al*. 1999).

Generally speaking, a wastewater treatment process can be divided in three steps. The primary treatment is a set of physical processes: first large debris are separated and grease removed from the influent, which is further clarified from

the organic suspended solids by sedimentation. The primary effluent is directed to the secondary treatment, where biological oxidation processes are carried out by microorganisms to remove the remaining suspended solids. The tertiary treatment comprises physicochemical processes to disinfect and remove turbidity of the final effluent, which is then discharged to water bodies or channeled for reuse (Figure 1.1).

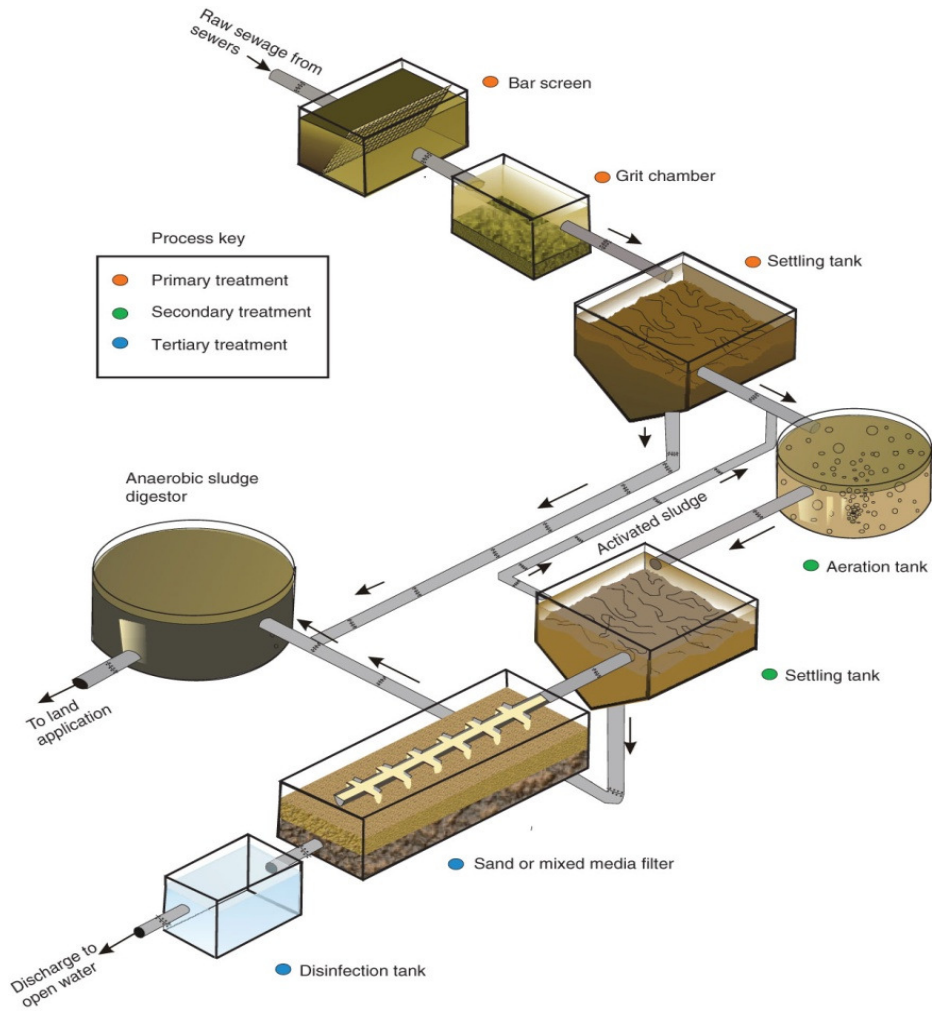

Figure 1.1: Schematic of a wastewater treatment plant. (Adapted from Maier *et al.* (2000) with permission)

The biological treatment of wastewater most commonly implemented is the conventional activated sludge process. The activated sludge is a flocculent suspended growth culture, rich in bacteria, in the primary treatment effluent, forming the mixed liquor suspended solids (MLSS) (Leslie Grady Jr *et al*. 1999). This treatment typically occurs in an aerobic tank where oxygen or air is provided, promoting cell growth and organic matter oxidation from the wastewater. In such conditions, the biomass grows in an aggregated form, forming suspended flocs of mixed microbial communities embedded in a matrix of extracellular polymeric substances (EPS). The wastewater pass through this matrix and the organic content, nutrients and pollutants are available for the microorganisms' uptake and use. The MLSS then flows into a secondary settling tank where the biomass and other suspended solids are separated from the effluent, which proceeds to the tertiary treatment before being discharged. This separation step relies on the capacity of the biomass to form flocs with relatively high settling velocity. Part of the sludge is wasted at this point, and directed to a sludge processing unit, whereas the remaining sludge is recirculated to the activated sludge tank (Maier *et al*. 2000).

1.1. Microbial Nutrient Removal from the Wastewater

The removal of nutrients such as phosphorus and nitrogen requires specific modifications of the conventional activated sludge system, with implementation of anoxic and/or anaerobic tanks and manipulation of operational parameters to promote the growth and performance of specific microorganisms.

1.1.1. Nitrogen removal

Nitrogen is present in the wastewater in the form of ammonia, discharged directly into the wastewater or resulting from the hydrolysis of e.g. urea and aminoacids. Ammonia is a toxic compound to aquatic species and a contributor to eutrophication of natural water environments. The successful mineralization of ammonia from wastewater is a two step process that encompasses nitrification and denitrification, transforming ammonia in gaseous nitrogen. Two functional groups of bacteria are involved in the biological nitrogen removal: the nitrifiers are autotrophic bacteria that use up ammonia (ammonia-oxidizing bacteria, AOB) or nitrite (nitrite-oxidizing bacteria, NOB); the denitrifiers are heterotrophic bacteria that reduce nitrate or nitrite to gaseous nitrogen (Leslie Grady Jr *et al*. 1999).

The nitrifiers, as all autotrophic bacteria, have a slow growth rate, as compared to heterotrophs, and AOB have a lower affinity for ammonium and oxygen than NH_4^+ assimilating heterotrophs. Hence, AOB are weak competitors for ammonium in activated sludge (Geets *et al*. 2006). They are also sensitive to toxic shocks, pH and temperature fluctuations and can be washed out with the waste activated sludge due to its slow growing rate and low concentration in the reactor at short sludge retention times. Consequently, many conventional wastewater treatment plants fail to establish a stable nitrification process (Zimmerman and Bradshaw 2004; Wagner and Loy 2002).

In fact, several biological techniques and processes have been developed and implemented for nitrogen removal from the wastewater. Conventional processes include the separation, in time or space, of the nitrification and

denitrification stages alternating and/or recirculating the influent between aerated and anaerobic tanks (Zhu *et al.* 2008).

1.1.2. Phosphorus removal

Phosphorus can be removed chemically or biologically from the wastewater. The chemical process involves the addition of coagulants to the wastewater and/or mixed liquor, resulting in the chemical precipitation of phosphorus together with other compounds and organic matter. This approach involves higher costs, not only due to the cost of the chemicals, but also because it produces large quantities of sludge, which have to be further processed (Seviour *et al*. 2003). The biological process, known as enhanced biological phosphorus removal (EBPR) overcome these disadvantages and is based on the circulation of the activated sludge between an anaerobic tank, where the sludge comes into contact with the wastewater influent, and a subsequent aerobic or anoxic tank (Smolders *et al.* 1994). In the latter case, concomitant removal of phosphorus and nitrogen is achieved. This cycling aims at the enrichment of the biomass with bacteria known to accumulate phosphorus in the form of polyphosphate granules, named polyphosphate accumulating organisms (PAOs). Simultaneous denitrification is performed by a group of PAOs (DPAOs) that can use nitrate or nitrite instead of oxygen as electron acceptor (Carvalho *et al*. 2007). Unlike ordinary heterotrophic microorganisms, PAOs are capable to take up volatile fatty acids under anaerobic conditions. Volatile fatty acids are taken up and stored intracellular as carbon polymers like poly-β-hydroxylalkanoate (PHA). This conversion uses the reducing power obtained by the glycolysis of internal storage glycogen and the energy obtained from the cleavage of the polyphosphate

granules, resulting in phosphorus release from the cell back into the wastewater. Under aerobic conditions, PAOs are capable of luxury uptake of phosphorus, and PHA polymers are degraded to acquire the energy for the conversion of phosphorus into polyphosphate granules, for the regeneration of the glycogen pools, and for biomass growth (Oehmen *et al*. 2007). Phosphorus is removed alongside with PAO bacteria with surplus sludge from the aerobic tank. The remaining sludge, after sedimentation, is recycled back to the aerobic tank. Despite the economic and environmental advantages of EBPR, this process poses more technical challenges than chemical precipitation, since it is prone to failure if not properly monitored. External disturbances, such as high rainfall, excessive nitrate loading and temperature, can negatively imply on the stability and reliability of EBPR. Another cause of EBPR failure is the presence of glycogen accumulating organisms (GAOs). PAOs can be out-competed by GAOs that grow in the same conditions and feed on the same carbon sources, but do not remove phosphorus from the wastewater. Therefore, GAOs are undesirable members of the microbial community of EBPR systems (Seviour *et al*. 2003).

1.2. Biological treatment by membrane bioreactors

Climate change is leading to global alterations in precipitation averages, which combined with population growth and the increasing extent of water contamination, can seriously endanger the availability of safe water in the near future. This problem can be mitigated through advanced wastewater treatment and water recycling, with systems based on membrane processes.

Membrane bioreactors (MBRs) are systems that combine the biological treatment process with a membrane that retains all the biomass (Judd 2006). MBRs present several advantages over conventional activated sludge process. The presence of a membrane that filters the water from the suspended solids in the system displaces the physical separation processes for sludge settling and tertiary disinfection process. Moreover, the process no longer relies on good settling properties of the sludge blanket for solid/liquid separation, thus it can be operated at higher biomass concentration levels, which further reduces the volume of the aeration tank. Both factors give MBRs a smaller footprint as compared to conventional activated sludge system. This is a very significant advantage taking into account the size and population density of the current cities. The total retention of solids permits to achieve higher sludge retention times, which favour the proliferation of slow growing microorganisms, which are important e.g. for biological nutrient removal. Finally, MBRs produce an effluent of high quality, without the need of further disinfection. This is a very attractive advantage of MBRs because the effluent can be directly discharged in the environment or it can be reused for non-potable applications, such as irrigation and recreational purposes (Le-Clech 2010).

The first MBRs were commercially developed in the sixties (Hardt *et al*. 1970). This first generation had a sidestream configuration, in which the membrane is located outside the reactor and the biomass is recirculated from the bioreactor to the membrane reactor. But this configuration was highly energy demanding due to the recirculating pump. In the beginning of the nineties, a new configuration came to reduce significantly the operational costs and boost the MBR technology application. In the second generation MBRs the membrane is located inside the bioreactor, in a submerged configuration. The driving force of

the water through the membrane is the transmembrane pressure (TMP), created by means of a suction pump.

Two types of membrane configurations are commonly applied in submerged MBRs: the flat sheet and the hollow fiber modules (Figure 1.2).

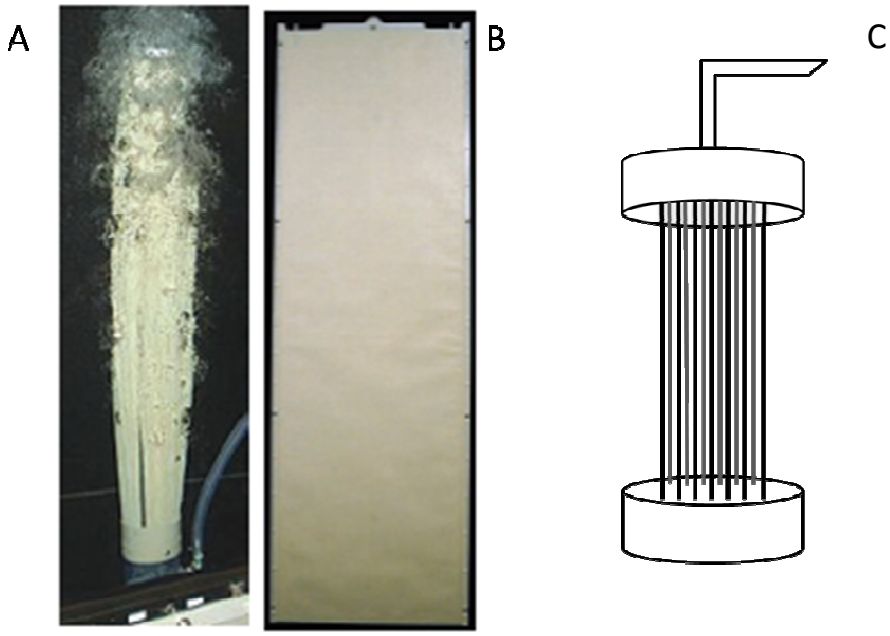

Figure 1.2: Membrane configurations most used in submerged MBRs. Pictures of A) Hollow fiber membrane module and B) flat-sheet membrane module. C) Schematic representation of a hollow fiber membrane module. Adapted from Judd (2006).

MBRs rely on the membrane pore size to obtain a clarified effluent. The commercially available membranes are generally polymeric membranes with pore sizes ranging from 0.02 to 0.5 µm (Le-Clech 2010; Yang *et al*. 2006). This means that MBRs can filter all the bacteria and colloids, as well as most of the viruses and

part of endotoxins (Judd 2006). Membranes can be polymeric (for example polyethylsulphone and polypropylene), ceramic and metallic (Judd 2006; Ramesh *et al*. 2006). Membranes made up by hydrophobic material can undergo surface modifications to become hydrophilic and interact poorly with the hydrophobic molecules of the MLSS (Judd 2006).

0.2.1. Operational parameters determining sludge characteristics

The proliferation of microorganisms inside a bioreactor is affected by the time that the biomass is retained in the system. In MBRs, with total retention of solids, sludge retention time can be set for long periods, even infinite. In such conditions the slow growing microorganisms may establish themselves in the bioreactor. The sludge retention time is one of the parameters with a direct impact on the ecophysiology of the biomass. By controlling the sludge retention time it is also controlled the substrate degradation rate (by permitting the operation of the MBRs at a low food to microorganisms (F/M) ratio), the biomass concentration (given by MLSS) and the excessive sludge production. The manipulation of the sludge retention time is done by sludge waste/purge (Judd 2006). When operating a MBR with high sludge retention time the MLSS raises. The operation at a high biomass concentration and low F/M ratio has several implications. The microbial cells are in a starvation state that makes them more readily available to remove the organic fraction from the wastewater, and the carbon is first used up for cell maintenance but not to cell growth (Witzig *et al.* 2002). Consequently there is a lower sludge production in comparison to conventional activated sludge process.

The first objective of aeration is to supply oxygen to the microorganisms, essential in activated sludge system for the microbial life cycle and removal of carbon and other nutrients from the wastewater. In MBRs aeration also performs the scouring of the membrane, removing part of the biomass from the membrane surface. This is one of the basic cleaning procedures to reduce membrane fouling, which will be discussed later. The aeration flow rate (Q_{aer}) also promotes mixed liquor agitation and high mass transfer rates. This is particularly important in high MLSS conditions where the viscosity of the mixed liquor increases, which implies in the dissolved oxygen (DO) availability in the sludge flocs (Judd 2006; Le-Clech 2010). In fact, the MLSS values applied in MBRs wastewater treatment are mainly limited by the system aeration capacity and by the costs associated to the amount of energy necessary for oxygen transfer in such conditions (Kraemer *et al.* 2012; van Nieuwenhuijzen *et al.* 2008).

1.3. Microbial populations present in WWTP

The initial studies of the microorganisms comprising the activated sludge applied classical microbiology isolation approaches through the cultivation of such environmental microorganisms, but gave very scarce outcomes, failing to achieve the true composition of complex communities (Wagner *et al.* 1993; Amann *et al.* 1995). The emergence of molecular techniques such as polymerase chain reaction (PCR), DNA fingerprint (DGGE and t-RFLP) and fluorescence *in situ* hybridization (FISH) allowed a first glimpse to the inside of the black box of who compose the activated sludge (Amann *et al.* 1995; Amann and Ludwig 2000). More recent molecular techniques, such as next generation sequencing, are starting to be

applied to the microbial populations of activated sludge and giving a clearer picture about the community structure.

1.3.1. General microbial population groups in WWTP

Activated sludge is dominated by prokaryotic microorganisms. Filamentous bacteria form the backbone of the activated sludge flocs, to which the floc formers bacteria attach (Raszka *et al.* 2006). The microorganisms are responsible for chemically convert the organic matter and nutrients. Thus, the efficiency and the stability of the performance of a WWTP is manly dependent on the composition and activity of such community.

The filamentous bacteria, up to a certain level, function as the backbone of the flocs structure, contributing to cell aggregation. Filamentous overgrowth, though, can pose problems such as foaming and poor settleability. Although in MBRs settleability of the biomass is overcome with the membrane filtration, foaming has the potential to pose performance issues to MBRs such as difficulties in controlling the MLSS in the aerobic/membrane tank, production of a foul odor and, in extreme cases, overflow the bioreactor limits (Di Bella and Torregrossa 2013). Filamentous bacteria in such conditions can produce and excrete extracellular polymeric substances with a high concentration of hydrophobic compounds, known to impact on the fouling of the membrane (Meng *et al.* 2007; Meng and Yang 2007). Several genera have been identified among the filamentous bacteria harbored by MBRs: *Chloroflexy*, *Nostocoida*, *Microthrix*, *Eikelboom* type 1851 and *Thiothrix* are among the most described in the literature (Di Bella and Torregrossa 2013; Miura and Okabe 2008).

The microbial communities are exposed to specific selective pressures in MBRs, different than those present in conventional activated sludge systems: 1) The biomass faces shear stress conditions due to the high aeration imposed for membrane scouring; 2) With the total retention of solids, all bacteria are maintained in the bioreactor and the diversity of the microbial population includes also those that are otherwise washed out in conventional activated sludge settlers; 3) The fact that MBRs are operated at higher MLSS can imply a higher competition for substrate between the communities.

Studies based on FISH analysis and DNA fingerprinting methods, such as denaturating gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (t-RFLP), have compared the microbial community between MBRs and conventional activated sludge systems, and all revealed significant differences between the population structures (Hall *et al.* 2010; Wan *et al*. 2011*;* Luxmy *et al. 2000;* Miura *et al.* 2007), even in systems fed with the same wastewater and subjected to very similar environmental conditions. In common between the two types of systems was the fact that *Proteobacteria* was the dominant group, from which *Betaproteobacteria* was the most frequent class of this phylum, making up almost half of the bacterial population, followed by *Alphaprotebacteria* (Miura *et al.* 2007; Luxmy *et al.* 2000). Apart from this, MBRs seem to select a more stable microbial community as compared to conventional activated sludge systems systems, where higher dynamics were observed (Hall *et al*. 2010; Wan *et al.* 2011). Moreover, a large set of novel and uncultured bacterial sequences have been found in a MBR (Wan *et al.* 2011), which reflects the lack of knowledge concerning MBRs' microbial populations.

1.3.2. Nutrient removal related communities

Nitrifying bacteria are divided into two microbial groups, concerning their role in the nitrification process in wastewater. AOB comprises two monophyletic groups, one included in the *Gammaproteobacteria* class and the other in the *Betaproteobacteria* class. Only the *Betaproteobacteria* AOB has been reported in WWTP, comprising the genera *Nitrosospira* and *Nitrosomonas*. This latter genus is recognized as the most important in performing ammonia oxidation in wastewater and the diversity of AOB species in WWTP is enormous (Wagner and Loy 2002). The NOB population includes the genera *Nitrobacter* and *Nitrospira*. Although *Nitrobacter* was traditionally considered an important nitrite oxidizer, in fact *Nitrospira* is the dominant NOB in most WWTP (Wagner and Loy 2002). *Nitrospira* have a high substrate affinity and low growth rate, as opposed to *Nitrobacter*, which have a low affinity to nitrite and oxygen. So, under substrate limiting conditions, *Nitrospira* can outcompete *Nitrobacter* and prevail in the activated sludge (Wagner and Loy 2002).

The microbial players in the removal of phosphorus from wastewater are commonly known as polyphosphate accumulating organisms, PAOs. *Candidatus Accumulibater phosphatis* is the most relevant organism of this group. *Accumulibacter* is a member of the *Betaproteobacteria,* closely related to the *Rhodocyclus* genus (Oehmen *et al.* 2007; Gebremariam *et al.* 2011). *Accumulibacter* was found in significant abundances in many lab scale and full scale EBPR systems, behaving accordingly to the described PAOs phenotype, but also in conditions not designed for phosphorus removal (Ahn *et al*. 2007; Pijuan *et al.* 2006). *Accumulibacter* is comprised by two clusters (I and II), each divided into several clades (IA-E and IIA-G) (He *et al.* 2007; McMahon *et al.* 2002). Metabolic

differences are found between the two types, like the denitrification capacity of members of the type I (Carvalho *et al.* 2007). Other putative PAOs have been described, such as *Dechloromonas*-related PAOs, similar to *Accumulibacter* concerning the capacity to take up volatile fatty acids and phosphate from the wastewater to perform internal reservoirs. However, performance in the EBPR context of these organisms needs further investigation (Kong *et al.* 2007); *Tetrasphaera*-related PAOs, belonging to the gram positive *Actinobacteria* phylum, are abundant in domestic and industrial wastewaters (Kong *et al.* 2005). *Tetrasphaera*-PAOs have a different phenotype from *Accumulibacter*, not fitting exactly in the profile conventionally described for EBPR (Gebremariam *et al.* 2011). Recently it was proposed that under anaerobic conditions these putative PAOs take up glucose as carbon source (Kristiansen *et al.* 2013). The glycogen is synthesized as a storage polymer, at the expense of their stored polyphosphate for energy for such conversion. During the aerobic phase, the stored glycogen is metabolized to provide energy for growth and to replenish the intracellular polyphosphate reserves. Currently *Accumulibacter* and *Tetrasphaera* are the two recognized players in the phosphorus removal from wastewater, but evidences from *in situ* studies for the detection of unidentified cells with polyphosphate granules demonstrate that the existence of others PAOs should be taking into account. The direct competitors of PAOs, globally named Glycogen Accumulating Organisms (GAOs), are phylogenetically diverse. *Candidatus Competibacter phosphatis* was the first GAO to be characterized (Crocetti *et al*. 2002). GAOs were described as abundant in EBPR plants with deteriorated biological phosphorus removal (Nielsen *et al*. 1999) and evidences were obtained that they directly compete with PAOs for volatile fatty acids uptake (Saunders *et al.* 2003). Other group of GAOs, belonging to the *Alphaproteobacteria* phylum, is related to

Defluviicoccus vanus and display a typical tetrad morphotype. *Defluviicoccus*-related organisms are divided into two monophyletic clusters and both have been demonstrated to compete with PAOs for anaerobic carbon uptake. Other microorganisms have been hypothesized as putative GAOs, such as *Comamonadaceae*-related *Betaproteobacteria* and *Thioalkalivibrio*-related *Gammaproteobacteria* (Kong *et al.* 2007), but the impact of such putative GAOs on phosphorus removal still needs further investigation.

Regarding nutrient removal from the wastewater, MBRs offer different conditions, such as operation of longer sludge retention time and complete retention of the biomass, as to the conventional systems. The specific MBRs conditions don't seem to impact on the diversity of the nutrient removal populations, in which the genera and functional groups are the same between conventional activated sludge systems and MBRs, but in their abundances, higher in MBRs (Saunders *et al.* 2013; Ma *et al.* 2013). These relations between the survival and proliferation of the microorganisms enrolled in nutrient removal and their performance in MBRs need further investigation to clarify the influence of the operational conditions on wastewater treatment.

1.3.3. Broad-coverage methodologies for microbial profiling

With the advance of metagenomics and application of technologies such as next generation sequencing, there was a substantial increase in the knowledge about the microbial populations of activated sludge. The principle of the metagenomic studies is to sequence an entire microbial community without isolating individual microorganisms, but rather sequencing a mixture of all the

community genomes. Although the bacterial diversity from conventional activated sludge systems has been the focus of metagenomic studies, the microbial community present in MBRs has just started to be addressed by such advanced molecular techniques. From the few MBRs metagenomic studies reported, part has looked into nutrient removal related microbial communities (Saunders *et al.* 2013; Ma *et al.* 2013) or the microbial composition under specific feed compositions (Zhu *et al*. 2013). The remaining studies applying next generation sequencing to MBRs addressed the involvement of the microorganisms on membrane fouling (Lim *et al.* 2012; Kim *et al.* 2013). Several conclusions can be summarized from these studies: the bacteria populations are very diverse and versatile, rapidly changing when confronted with changes in environment in a MBR (Lim *et al*. 2012; Zhu *et al.* 2013). In agreement with former studies, *Proteobacteria* are the most dominant phylum in MBRs, but most of these novel studies conclude that, contrarily to what was described using FISH analysis and DNA fingerprinting methods, the *Gammaproteobacteria* is the most important taxonomic class, but there is no agreement about the relevance of *Betaproteobacteria* (Zhu *et al.* 2013; Kim *et al.* 2013; Lim *et al.* 2012). Also, the bacterial structures from the mixed liquor and the fouling layer of the membrane, the cake layer, are different (Lim *et al.* 2012; Kim *et al.* 2013). The fouling layer offers different conditions and selective pressures to the bacteria then the mixed liquor. The affinity to the membranes, the diffusion of the oxygen in a more compact biomass and the membrane scouring by the aeration are features that can affect bacterial selection in the cake layer. Regarding the core microbial groups participating in nutrient removal, they are mostly coincident in conventional activated sludge systems and MBRs systems (Saunders *et al.* 2013; Ma *et al.* 2013). However, MBRs presented a higher diversity of other bacteria,

such as the fermentative bacteria and polymer degraders, not described in conventional activated sludge systems (Saunders *et al.* 2013).

Despite the increasing information available about the microbial communities in MBRs, there are still many questions to be explored. The impact of the operational conditions and specificities of MBRs, such as the sludge retention time, aeration, membrane flux and membrane material, on the microbial diversity and structure is yet to be explored, which could contribute to understand and solve failures in MBRs performance and wastewater treatment.

1.4. Extracellular Polymeric Substances

In nature, the majority of the microorganisms lives and grows in an aggregated form. Extracellular polymeric substances (EPS) are the matrix that maintains the cells together for the microorganisms to live in the form of biofilms, flocs and granules. However, EPS have other functions beyond aggregation: it acts as a protective matrix from external aggressions (dewatering, toxic substances, high temperature and pH) and provide means for easy access to nutrients, for genetic information exchange and for extracellular enzymatic reactions (Flemming and Wingender 2010; Sheng *et al*. 2010; Wingender *et al.* 1999). EPS can even be regarded by some bacteria, for example by the filamentous *Chloroflexi*, as a food source (Miura and Okabe 2008).

EPS by definition are located at the membrane surface or outside it. The composition of EPS results from different processes: active secretion, shedding of cell surface material, cell lysis and adsorption from the environment. In the context of activated sludge Nielsen *et al*. (1997), subdivided EPS as soluble and

bound EPS. Bound EPS are closely bound with cells and settles together with activated sludge while soluble EPS are weakly bound to cells or dissolved in the solution, being a component of the effluent (Sheng *et al.* 2010; Raszka *et al.* 2006).

EPS are a highly hydrated matrix, where cells are embedded, by forming a polymeric network maintained by several types of weak intermolecular interactions such as ion bridging, hydrogen bonding and electrostatic attractive forces (Mayer *et al.* 1999). EPS are composed by several macromolecules, among which proteins and polysaccharides are the major components. Early studies on EPS pointed out polysaccharides as the predominant constituent (Wingender *et al*. 1999). However, this statement was based on EPS extracted from pure cultures. In fact, the EPS of mixed culture systems, such as activated sludge, seems to be dominated by proteins (Liu and Fang 2003). Other constituents include humic acids, lipids and extracellular DNA (eDNA). The distribution of those components is heterogeneous along the matrix.

Studies about extracellular enzymatic activity and with biofilms of pure cultures have revealed that the proteins present in the EPS have two functions: enzymatic and structural (Eboigbodin and Biggs 2008; Flemming and Wingender 2010; Wingender *et al*. 1999). Extracellular enzymes are retained in the matrix by interactions with polysaccharides and are responsible for the breakdown of biopolymers into smaller molecules that can be taken up and utilized as carbon and energy source by the microorganisms. Other enzymes, such as lyases and hydrolases, are degraders of EPS components promoting the dispersion of the sessile cell as a response to environmental pressure. Some classes of enzymes have been described as extracellular in the biofilm context including proteases,

peptidases, glucosidase, glucoronidase, lipase, phosphatase and oxireductase (Xia *et al.* 2008; Flemming and Wingender 2010; Wingender *et al.* 1999). Apart from the enzymatic function, proteins also behave as structural elements of the biofilms, together with the polysaccharides and the eDNA. They perform the link between the cells and the EPS matrix and can be cell associated and polysaccharide associated (Flemming and Wingender 2010). Proteins interact by means of their negatively charged aminoacids with divalent cations and are mainly composed by hydrophobic aminoacids; consequently they are important in the formation and cohesion of flocs, being key elements for the flocculation, settling and dewatering of the activated sludge (Sheng *et al.* 2010; Wingender *et al.* 1999; Higgins and Novak 1997).

The identification of EPS proteins forming the suspended flocs of activated sludge has been long attempted due to its importance on the biofloculation process and in the extracellular metabolism of complex organic substrates (Albertsen *et al.* 2013; Park *et al.* 2008; Higgins and Novak 1997; Denecke 2006; Kuhn *et al.* 2007). However, the number and diversity of the extracellular proteins presently known is low, as all studies, despite the advanced proteomics technology available, have identified only few proteins from the mixed liquor EPS. Several technical difficulties have been encountered, namely the resolution of protein bands in gel and the number of proteins retrieved from mass spectrometry analyses. More recently, Albertsen *et al.* (2013) combined the use of metagenomics and proteomics to increase the knowledge about the protein fraction of the EPS layer: the search of genes encoding for known biofilm proteins from pure culture studies had a scarce outcome, only finding eight protein candidates; from the proteomic analysis only two proteins were found.

Several obstacles are found when EPS proteins are to be characterized: the complexity of the EPS matrix likely hinders protein purification from other macromolecules; the peptide concentration to be analyzed by mass spectrometry is low; and the search for protein similarity in the databases for its identification requires entries of proteins from closely related organisms, which often fails in the activated sludge case, primarily dominated by uncultured or unidentified bacteria (Albertsen *et al.* 2013; Park *et al*. 2008; Higgins and Novak 1997; Denecke 2006; Kuhn *et al*. 2007).

Several factors can implicate in the variation of the composition of the EPS, such as sludge retention time, the type of wastewater, nutrient level and others. Sludge retention time impacts on the microbial structure and activity of the activated sludge, and therefore in the EPS produced. But more that being implicated in the amount of EPS produced, sludge retention time has an effect on the proportion of the EPS components. Longer sludge retention times resulted in an increase in the protein to polysaccharide ratio (Sheng *et al.* 2010; Liao *et al*. 2001). The activated sludge also responds to external factors, like toxic conditions, with an overexpression of EPS as a form of protection. In these conditions, proteins were the constituent with higher concentration increase (Avella *et al.* 2010; Sheng *et al.* 2005).

EPS have several characteristics important for wastewater treatment. EPS has the capacity to adsorb metals and organic molecules by the presence of hydrophobic regions and negatively charged functional groups, which gives EPS a high binding capacity. In the flocculation process, divalent cations, such as Ca^{2+} and Mg^{2+} interact with the negatively charged functional groups of the polymers, acting as a bridging element between EPS molecules of the different microbial

cells. EPS are amphoteric, in which proteins are the main hydrophobic components and polysaccharides are responsible for the hydrophilic characteristics (Liu and Fang 2003; Sheng *et al.* 2010). EPS has the ability to adhere to a solid surface by several possible interactions, namely electrostatic, chemical, ion bridging and polymeric. The adherence of EPS to a surface alters its characteristics and paves the way to bacterial colonization (Tong *et al.* 2010; Raszka *et al.* 2006).

1.4.1. Membrane fouling

The major obstacle for the wide application of MBRs in the treatment of wastewater is the rapid decline of the permeate flux (J) over time as a result of membrane fouling. A significant part of the scientific research concerning MBRs is related to fouling (van Nieuwenhuijzen *et al.* 2008; Yang *et al.* 2006). This global interest is due to fouling impact in filtration efficiency, which reduces MBRs performance, shortening the membrane lifespan and increasing operation costs.

Fouling of the membrane was defined in Drews (2010) as the coverage of the membrane surface (external and internal) by deposits which adsorb or simply accumulate during operation. Fouling can be due to inorganic depositions, as by calcium carbonate scaling (van Nieuwenhuijzen *et al*. 2008), or have a biological origin (Meng 2009). The membrane is in direct contact with the activated sludge and cells, colloids and EPS can deposit on the membrane surface and inside its pores, decreasing its permeation capacity (Drews 2010; Judd 2006).

Fouling can be classified in function of permeability recovery (Guo *et al.* 2008; Meng *et al.* 2009; Judd 2006) as:

- removable, caused by loosely attached foulants, when it can be removed by physical cleaning, like backwash or relaxation of the permeation;
- irremovable fouling, caused by pore blocking and strongly attached foulants, which is only removed by chemical cleaning like sodium hypochlorite;
- irreversible fouling which results from the build up of fouling over the operation that is not removed by any of the described means

Transmembrane pressure (TMP) is often used as a fouling indicator, since the accumulation of fouling agents on the membrane surface induces an increase in the resistance to permeation across the membrane, resulting in an increase in TMP. This effect can be described as a three step phenomenon (Meng *et al.* 2009; Wang and Wu 2009):

1st: An initial and rapid increase in the TMP due to the first contact of the membrane with the mixed liquor. Colloids and soluble molecules deposit at the surface of the membrane and inside its pores, forming the gel layer.

2nd: A slow increase in the TMP is observed throughout operation time. The permeation flux drives sludge flocs and bound EPS towards the membrane. A cake layer starts to form by biomass compression and colonization of the membrane surface, aided by adhesion onto the gel layer (Chu and Li 2005).

3rd: A sharp increase in the TMP occurs, consequence of severe membrane fouling, with influence of the increasing release of EPS related to the consolidation of the cake layer and oxygen transfer limitations inside the cake.

The sequential formation of two different layers is dependent on the velocity of the flux across the membrane.

EPS are regarded as the major foulant in MBRs. The two fractions of EPS have been implicated in the fouling of the membrane. Soluble EPS, composed of colloidal and soluble matter, are described as the principal responsible for the formation of the gel layer by adherence and deposition of molecules at the surface and inside the pores of the membranes (Wu *et al*. 2013; Wang and Wu 2009; Viero *et al.* 2007; Huang *et al.* 2011). The rejection of particles by the membrane is ruled by the pore size; in ultrafiltration and microfiltration membranes polysaccharides are typically rejected to a greater extent than proteins, thus being retained at the surface of the membrane (Drews 2010). This initial deposition of EPS, as already mentioned, facilitates the following deposition of bound EPS molecules and cell colonization. The transport of sludge flocs to the vicinity of the membrane due to the suction forces enhances this colonization and leads to the formation of the cake layer. However, fouling is dependent on MBRs proprieties, such as membrane material and operational conditions and aeration turbulence is applied in MBRs to minimize the accumulation of sludge flocs at the membrane surface (Wu *et al*. 2013).

Bound EPS are the major contributor for the cake layer formation and resistance (Wang and Wu 2009; Cho *et al.* 2005). Cake layer resistance was described as the major responsible for the total resistance to the permeate flux,

and cake layer formation is regarded as an important factor for membrane fouling (Meng and Yang 2007). However, some researchers have found that, at the beginning of the cake layer formation (as a thin layer), the cake formed the equivalent to a second “membrane”, which is referred as a dynamic membrane, at the surface of the MBR membrane (Zhang *et al.* 2010; Lee et al. 2001). The cake membrane is described to act as a sieve, screening the primary membrane from strong fouling species, such as the soluble EPS foulants. With consolidation of the cake this feature of dynamic membrane is no longer relevant and fouling of the membrane occurs with the increase of the filtration resistance and decrease of the membrane permeability (Wang and Wu 2009). It was suggested that the EPS in the cake layer is heterogeneous regarding its composition as well as the molecular distribution along the cake layer depth (Gao *et al.* 2011) and proteins have been described as the major EPS component of the cake layer (Hu *et al.* 2013).

Some operational conditions than have direct implication on the biomass characteristics have been studied regarding the fouling of the membrane. Sludge retention time is one of the most important operational parameters affecting MBRs fouling. In general, lower membrane fouling rates are observed at elevated sludge retention time due to the decrease in EPS concentration, a better flocculation of the biomass and a higher stability of the sludge flocs (Tian *et al.* 2011; Sweity *et al*. 2011; Van den Broeck *et al.* 2010; Meng *et al*. 2009, Ahmed *et al.* 2007). But this trend seems to be observed only in a certain range of sludge retention times (Han *et al.* 2005; Lee *et al.* 2003). Wu *et al*. (2011) compared three different sludge retention times (10 d, 30 d and infinite) and verified that at

infinite sludge retention time, the cake fouling was maximum due to the increase of the EPS content, principally in the bound EPS.

Although polysaccharides have been pointed out as the major foulants in soluble EPS, recently proteins have been also described as important fouling agents (Tian *et al.* 2011; Tang *et al.* 2010; Miyoshi *et al.* 2009). To a better knowledge and control of the fouling mechanisms it is important to gain information about the profiles of each of the EPS constituents, but very few studies have addresses this issue. Huang *et al.* (2012) has described EPS proteins species found in the gel layer extracted from membranes of different materials. The different characteristics of the membrane material, such as the hydrophobicity and surface charge, were described to affect membrane fouling. From protein identification carried out in this study, several conclusions were drawn: there are proteins that adhere specifically to certain membrane materials; membranes attracted proteins with similar hydrophilic/hydrophobic nature; and the charge of the proteins does not seem to contribute to their adhesion to the different membrane' materials tested. Several proteins were obtained from the different membranes and common to all was the predominance of the 60 kDa chaperonin, known to be expressed in stressful conditions. Miyoshi *et al*. (2012) also sequenced proteins from the gel layer of hollow fiber and flat sheet membranes operated at different sludge retention times. The membranes operated at longer sludge retention time revealed less fouling. From both reactors 32 protein spots were observed in gel. However, amino acid sequences were only obtained for 11 proteins, from which only two were identified by database search, matching the outer membrane proteins OprF and OprD from *Pseudomonas* genus.

Due to the characteristics of specific EPS proteins that permit to adhere to membrane surfaces and to contribute to the cohesion of the EPS matrix, it is important to further investigate the protein species present on fouling layers, in order to design better strategies to control the fouling in MBRs.

1.5. Objectives and layout

A high quality effluent can be achieved in MBRs due to the total retention of solids by the membrane, the high sludge retention time that leads to the proliferation of slow-growing organisms, and the high biomass concentrations that enable the reduction of hydraulic retention time. These and other specific MBRs features likely affect the microbial population structure and ecophysiology. This thesis investigated the microbial communities developed in MBRs operated in different conditions, and their link with process performance, including the EPS, known as key fouling agents. Within the EPS, special focus was given to proteins, which have a high complexity of possible functions that are essential for aggregate structure, adhesion and activity.

The approach used to investigate the microbial community structure and extracellular proteome of MBRs was based on molecular biology tools.

This study was divided in three steps:

1) Investigation at the microbial level if MBRs favor growth and performance of nutrient removal related organisms.

2) Analysis of the microbial populations and associated extracellular proteomic profile and dynamics at different operation conditions
    a. Optimization of the methodology for extracellular proteome identification
    b. Analysis of the microbial ecology and extracellular proteome at different conditions
3) Characterization of the biofouling agents by membrane autopsy

This thesis is divided into six chapters:

- **Chapter one** is a general introduction concerning the microbiology of activated sludge, including the microbial populations and extracellular polymers with special focus on the impact of MBRs on the microorganisms' ecophysiology. Emphasis is given to the organisms responsible for phosphorus removal from the wastewater and to the EPS proteins expressed in certain conditions.

- **Chapter two** concerns the characterization of the microbial populations enrolled in biological nutrient removal from the wastewater and their proliferation on MBRs. The nitrifying community structure in MBRs described by others was confirmed. A comprehensive characterization of the populations related to phosphorus removal, inexistent in literature thus far, was carried out. Conclusion were drawn concerning the capacity of these microorganisms of thrive in MBRs and their corresponding activity,

taking into account if the plants were designed or not for enhanced biological phosphorus removal.

- **Chapter three** described a workflow proposed to overcome several obstacles encountered in the study of the proteins from the extracellular proteome of activated sludge. This workflow comprises a number of steps that culminate in the identification of a high number of different proteins from bacterial origin from the soluble and bound EPS of suspended biomass flocs. This strategy has a high potential of application not only to the activated sludge of MBRs, but also to study the EPS proteins of other environmental mixed cultures.

- In **chapter four**, the strategy defined in chapter three was applied to characterize the EPS proteins of a lab-scale MBRs subjected to two different sludge retention times. Simultaneously, the corresponding microbial population, still fairly unknown in MBRs, was profiled by high throughput sequencing and the influence of MBRs specific operational conditions on the community structure was assessed. Also, the community profile of the 15 most important OTUs found in the MBRs mixed liquor and fouling layer were compared and differences were reported. The identification of EPS proteins from the mixed liquor of MBRs was presented and correlated with the corresponding operational conditions.

- **Chapter five** presents the characterization of the main EPS foulants responsible for the formation of the gel and cake fouling layers in a

lab-scale MBRs. Several methods were tested for the best coverage of the macromolecules extracted from the gel layer through membrane autopsy. The importance of the EPS molecules in each layer was discussed, and the identification of the EPS proteins from the cake layer was also described.

- **Chapter six** presents an overview discussion of all the conclusions from the thesis results and presents perspective knowledge gaps to be addressed in future work.

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CHAPTER 2

Microbial population analysis of nutrient removal-related organisms in membrane bioreactors

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Silva AF. was involved in all the experimental work presented in this chapter, except for the operation of the membrane bioreactors, biomass sampling and fixation, and data compilation (performed by Lousada-Ferreira M, van Nieuwenhuijzen A).

CONTENTS

Abstract

Membrane bioreactors (MBR) are an important and increasingly implemented wastewater treatment technology, which are operated at low food to microorganism ratios (F/M) and retain slow growing organisms. Enhanced biological phosphorus removal (EBPR)-related organisms grow slower than ordinary heterotrophs, but have never been studied in detail in MBRs. This study presents a comprehensive analysis of the microorganisms involved in EBPR in pilot- and full-scale MBRs, using fluorescence *in situ* hybridization (FISH), as well as an overall assessment of other relevant microbial groups. The results showed that polyphosphate accumulating organisms (PAOs) were present at similar levels in all studied MBRs (10 ± 6%), even those without a defined anaerobic zone. Glycogen accumulating organisms (GAOs) were also detected, although rarely. The FISH results correlated well with the observed P-removal performance of each plant. The results from this study suggest that a defined anaerobic zone is not necessarily required for putative PAO growth in MBRs, since polyphosphate storage may provide a selective advantage in fulfilling cell maintenance requirements in substrate-limited conditions (low F/M).

2.1. Introduction

Membrane bioreactors (MBRs) are an increasingly important technology for the treatment of wastewater (Judd 2008). The presence of a membrane that completely retains the solids of the mixed liquor obviates the presence of secondary settlers in a conventional activated sludge (CAS) wastewater treatment plant (WWTP). Thus, MBRs lead to a reduced footprint and high effluent quality (Le-Clech 2010).

The microbial composition of MBRs is still largely unknown, and the presence of a membrane and other specific MBRs operational conditions are new selective pressures for the microbial community as compared to CAS systems. The total retention of solids in MBRs implies that all microorganisms are retained in the biological tank (except for the biomass purged), as opposed to CAS, where the microbial populations with lower settling capacity are washed out from the clarifiers (Le-Clech 2010).

A few studies have compared the microbial community between MBR and CAS operated in parallel and all revealed significant differences between the overall community structures (Luxmy *et al.* 2000; Hall *et al.* 2010; Wan *et al.* 2011). MBRs seem to select a more stable microbial community as compared to CAS systems, where higher dynamics were observed (Hall *et al.* 2010; Wan *et al.* 2011). Moreover, a large set of novel and uncultured bacterial sequences have been found in an MBR (Wan *et al.* 2011), which reflects the lack of knowledge concerning MBR microbial populations.

Previous studies have investigated biological nutrient removal (BNR) in MBRs, mainly focusing on the nitrification processes and the populations involved.

The same groups of nitrifiers have generally been found in MBRs and CAS. Others groups of bacteria involved in BNR have been very scarcely characterized in MBR systems, such as polyphosphate accumulating organisms (PAOs). PAOs store P in the form of intracellular polyphosphate granules, which is typically achieved by recirculating the activated sludge between anaerobic and aerobic conditions, a process known as enhanced biological phosphorus removal (EBPR). EBPR is a well-accepted process but prone to failure. Glycogen accumulating organisms (GAOs) grow under the same conditions as PAOs and compete with them for anaerobic uptake of carbon sources, but do not remove phosphorus from the wastewater (Oehmen *et al.* 2007; Seviour and McIlroy 2008).

Other EBPR studies on MBRs have mainly focused on the optimization of operational conditions for P-removal, using chemical analysis to assess the activity of PAOs (Lesjean *et al.* 2005; Parco *et al.* 2007; Monclus *et al.* 2010). In MBRs, to the best of our knowledge, only Fu *et al.* (2009) have studied the presence, though not the abundance, of one type of PAO (*Accumulibacter*) in an anoxic/oxic pilot-scale MBR. The abundance of *Accumulibacter* and other microorganisms relevant in EBPR processes, such as other PAO microbial groups (i.e. *Tetrasphaera*-related and *Dechloromonas*-related) and GAOs (i.e. *Competibacter* and *Defluviicoccus*-related), have not previously been studied.

Although PAOs thrive under anaerobic/aerobic conditions, they do not necessarily require these operational conditions in order to survive, persisting in bioreactors operated under strict aerobic conditions as well as other aquatic habitats (Pijuan *et al.* 2006; Peterson *et al.* 2008). MBRs present a potentially suitable environment for PAOs proliferation. PAOs grow slower than ordinary heterotrophic organisms (Smolders *et al.* 1994), thus being favoured in MBRs due

to complete biomass retention (Hall *et al.* 2010). Additionally, the high biomass concentrations normally found in MBRs might lead to areas of anaerobic micro-niches within the sludge flocs in poorly mixed zones, potentially providing PAOs a selective advantage. Nevertheless, the presence of putative PAOs alone does not necessarily imply EBPR activity, which will depend on the operational conditions. Linking the microbial population with the BNR performance achieved in MBRs was the motivation for the present study.

This study characterized the microbial diversity of the activated sludge in a group of eight MBR plants fed with municipal wastewater, located in different regions of Europe. Particular emphasis was given to the populations involved in phosphorus removal, in view of the lack of information about the presence of EBPR-related organisms in MBRs. The abundance of putative PAOs and GAOs was determined through a large set of previously designed probes targeting these microorganisms, and related to the P-removal observed in each plant. This information can contribute to a better understanding of the potential of MBRs to achieve biological P-removal.

2.2. Material and Methods

2.2.1. MBR plants

Eight MBR plants fed with real wastewater were studied. In all plants the membrane was submerged in a separate tank from the main biological tank(s). Four MBRs were pilot-scale systems and four were full-scale plants (see plant locations in Table 2.1).

Table 2.1: MBR design data

MBR plant	Location	Scale	Design	Biological tanks (m^3)	Membrane tank (m^3)	Membrane type[a]	Total membrane area (m^2)	Membrane pore size (µm)	SAD_p ($m^3 m^{-3}$)[b]	Recirculation ratio[c]
MBR 1	Monheim, Germany	Full	Non EBPR	680 (aerobic); 680 (anoxic)	300	HF (Zenon Zee Weed 500c)	12320	0.040	12.5-36.0	11.0-53.0
MBR 2	Nordkanal, Germany	Full	Non EBPR	2609 (anoxic); 916 (aerobic/anoxic)	5784	HF (Zenon Zee Weed 500c)	84480	0.040	17.0	4.0
MBR 3	Schilde, Belgium	Full	Non EBPR	500 (anoxic); 500 (aerobic)	240	HF (Zenon Zee Weed 500c)	10560	0.040	10.0-17.2	5.8
MBR 4	Heenvliet, The Netherlands	Full	EBPR	391 (total)	152	FS (Toray)	4110	0.080	12.3	2.0
MBR 5	Margarethenhöhe, Germany	Pilot	EBPR	0.6 (anaerobic); 4.0 (aerobic); 4.0 (anoxic)	0.6	FS (Martin Systems and A3)	69	0.035 (Martin Systems); 0.2 (A3)	20.5	4.0
MBR 6	Trondheim, Norway	Pilot	Non EBPR	0.063 (each of 4 tanks)	0.033	HF (Zenon Zee Weed 500c)	3.72	0.040	18.7	n.a.
MBR 7	Zurich, Switzerland	Pilot	Non EBPR	0.5 and 4.0 (aerobic tanks)	1.6 (Zenon); 1.4 (Kubota); 0.6 (Puron)	HF (Zenon); FS (Kubota); HF (Puron)	116	0.040	22.0-73.0	n.a.
MBR 8	Lavis, Italy	Pilot	Non EBPR	5.0 (anoxic); 9.2 (aerobic)	1.5	HF (Zenon Zee Weed 500c)	70	0.040	17.0-20.0	4.0

[a]HF: hollow fiber; FS: flat sheet; [b]SAD_p: specific aeration demand per permeate flow; [c]recirculation between the membrane and biological tanks; n.a.: not available

MBR5 had two flat sheet-type membrane modules with different pore sizes in the membrane tank and MBR7 contained three parallel membrane tanks with different types of membranes. MBR4 and MBR5 were the only two plants specifically designed for EBPR with well-defined anaerobic zones. Details about the design of the MBRs are given in Table 2.1.

2.2.2. Collection of biomass samples and operational data

Biomass samples were collected from the membrane tanks and fixed with 4% paraformaldehyde for gram negative bacteria or with ethanol for gram positive bacteria (Amann 1995). The corresponding operational parameters and nutrient removal data are shown in Table 2.2. It should be noted that MBR1 and MBR2 apply chemical precipitation, thus the P-removal in these systems was not only attributable to biological activity.

Table 2.2: Operational data of the full- and pilot-scale MBRs studied within approximately one SRT (except otherwise indicated)

MBR plant	HRT (h)	SRT (d)	MLSS (g L^{-1})	Flux (L m^{-2} h^{-1})	CODt influent (mg L^{-1})	CODt permeate (mg L^{-1})	NH_4-N influent (mg L^{-1})	NH_4-N permeate (mg L^{-1})	P influent (mg L^{-1})	P permeate (mg L^{-1})	P removal (mg L^{-1})	Influent COD/P ratio (mgCOD mg^{-1}P)	F/M (gCOD g^{-1}MLSS d^{-1})
MBR 1	16.6	40	11.5	8.1	447	15.0	26.0	0.20	7.3 (Pt)	0.50 (Pt)	6.8	61	0.06
MBR 2	4.1	27	12.0	27.0	578	25.2	48.1	0.01	7.5	0.32	7.2	77	0.28
MBR 3[a]	3.6	21	11.0	33.0	175	27.0	17.0	<1.00	9.3	1.00	8.3	19	0.11
MBR 4	4.9	20	12.0	24.3	157	26.0	100	0.10	27.0	0.90	26.1	6	0.06
MBR 5	14.3	25	16.4	20.2	1178	45.0	120	0.10	16.8	0.10	16.7	70	0.09
MBR6[b]	2.0	1	0.8	35.0	247	28.8	21.3	15.80	6.5	n.a.	n.a.	38	3.70
MBR 7	2.1	26	10.0-15.0	19.3	388	18.3	19.0	0.70	2.0	2.90	-0.9	194	0.35
MBR8	7.9	22	8.6	28.4	505	47.7	40.1	0.40	3.3	2.30	1.0	153	0.18

[a]Single sample; [b]within one week; n.a.: not available; Pt: total P.

2.2.3. Microbial community characterization by FISH analysis

FISH analysis was conducted according to Amann (1995). The oligonucleotide probes used are listed in Table 2.3. Several probes were applied together or sequentially: PAO462, PAO651 and PAO846 (PAOmix); EUB338, EUB338-II and EUB338-III (EUBmix); GB-G2 and GAOQ989 (GAOmix); TFO_DF218 and TFO_DF618 (TFOmix); DEF988 and DEF1020 (DEFmix); Actino221 and Actino658; and NSO1225 and NSO190. The general probes for *Bacteria* (EUBmix) were used together with the specific probes for microbial population characterization and quantification purposes. *Archaea* were visualized against 4',6-diamidino-2-phenylindole (DAPI). The 5' labeling of EUBmix was either fluorescein isothiocyanate - FITC (epifluorescence microscopy) or cyanine 5 - Cy5 (confocal microscopy), while the specific probes were Cy3 -labelled. Unless otherwise specified, the probe details can be found in Nielsen *et al.* (2009).

Semi-quantification of *Archaea* and general bacterial groups commonly present in WWTP (*Alpha-, Beta-* and *Gamma-Proteobacteria* and *Actinobacteria*), as well as AOBs and NOBs, was carried out using a Leica DMRA2 epifluorescence microscope. Preliminary semi-quantification of the PAO and GAO populations was also performed. The EBPR-related populations showing >1% of apparent abundance were then quantified using a ZEISS LSM510/META confocal laser scanning microscope (CLSM) through the analysis of at least 30 images with the softwares Zeiss LSM Image Browser and ImageJ. Quantification values are given as biovolume abundance with respect to the EUBmix signal. The standard error of the mean (SE_{mean}) was calculated as the standard deviation divided by the square root of the number of images.

Table 2.3: Oligonucleotide FISH probes sequences and target sites

	Probe	Sequence (5' - 3')	Target
Higher taxonomic levels	EUB338	GCTGCCTCCCGTAGGAGT	Most *Bacteria*
	EUB338-III	GCAGCCACCCGTAGGTGT	*Planctomycetales* and other *Bacteria* not detected by EUB338
	EUB338-II	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales* and other *Bacteria* not detected by EUB338
	ALF969[a]	TGGTAAGGTTCTGCGCGT	*Alphaproteobacteria*
	BET42a	GCCTTCCCACTTCGTTT	*Betaproteobacteria*
	GAM42a	GCCTTCCCACATCGTTT	*Gammaproteobacteria*
	HGC69a	TATAGTTACCACCGCCGT	High G+C content Gram-positive *Bacteria* (*Actinobacteria*)
	Arc915[b]	GTGCTCCCCCGCCAATTCCT	*Archaea*
PAO	PAO462	CCGTCATCTACWCAGGGTATTAAC	Most *Accumulibacter phosphatis*
	PAO651	CCCTCTGCCAAACTCCAG	Most *Accumulibacter phosphatis*
	PAO846	GTTAGCTACGGCACTAAAAGG	Most *Accumulibacter phosphatis*
	Acc-I-444[c]	CCCAAGCAATTTCTTCCCC	*Accumulibacter phosphatis* clade IA
	Acc-II-444[c]	CCCGTGCAATTTCTTCCCC	*Accumulibacter phosphatis* clades IIA, C and D
	Actino221	CGCAGGTCCATCCCAGAC	*Tetrasphaera*-related *Actinobacteria*
	Actino658	TCCGGTCTCCCCTACCAT	*Tetrasphaera*-related *Actinobacteria*
	Bet135[d]	ACGTTATCCCCCACTCAATGG	*Dechloromonas*-related *Betaproteobacteria*

Table 2.3: Oligonucleotide FISH probes sequences and target sites (continuation)

	Probe	Sequence (5' - 3')	Target
GAO	GAOQ989	TTCCCCGGATGTCAAGGC	Some Competibacter phosphatis
	GB_G2	TTCCCCAGATGTCAAGGC	Some *Competibacter phosphatis*
	TFO_DF218	GAAGCCTTTGCCCCTCAG	*Defluviicoccus vanus*-related *Alphaproteobacteria* cluster 1
	TFO_DF618	GCCTCACTTGTCTAACCG	*Defluviicoccus vanus*-related *Alphaproteobacteria* cluster 1
	DF988	GATACGACGCCCATGTCAAGGG	*Defluviicoccus vanus*-related *Alphaproteobacteria* cluster 2
	DF1020	CCGGCCGAACCGACTCCC	*Defluviicoccus vanus*-related *Alphaproteobacteria* cluster 2
	Bet65[d]	CAGTTGCCCCGCGTACCG	*Comamonadaceae*-related *Betaproteobacteria*
	Gam455[d]	CTGACGTATTCGGCCAGTGC	*Thioalkalivibrio*-related *Gammaproteobacteria*
AOB	NSO1225	CGCCATTGTATTACGTGTGA	*Betaproteobacterial* ammonia-oxidizing bacteria
	NSO190	CGATCCCCTGCTTTTCTCC	*Betaproteobacterial* ammonia-oxidizing bacteria
NOB	NIT3	*CCTGTGCTCCATGCTCCG*	*Nitrobacter spp*
	Ntspa662	GGAATTCCGCGCTCCTCT	Genus *Nitrospira*

[a]Oehmen *et al.* 2006; [b]Stahl and Amann 1991; [c]Flowers *et al*. 2009; [d]Kong *et al.* 2007

2.3. Results

2.3.1. General microbial characterization

The epifluorescence microscopic analysis revealed common features in all MBRs studied: the diversity of cellular morphology was high and many different cell types could be found dispersed in the flocs or grouped in clusters with different sizes and shapes. Filamentous bacteria (of particularly large size in MBR 7, see Figure S2.1) were the backbone of the flocs, together with an abundant autofluorescent matrix, likely composed of extracellular polymeric substances (EPS).

In all of the MBRs, the dominant *Bacteria* group was the *Betaproteobacteria*, followed by the *Gammaprotebacteria*. *Actinobacteria* (high G+C content Gram positive bacteria) were also observed in all plants, usually in higher abundance than the *Alphaproteobacteria* (Table 2.4). Overall, the general community characteristics in the studied plants were similar to previously reported studies on MBRs, with *Betaproteobacteria* as the most abundant group (Luxmy *et al.* 2000; Witzig *et al.* 2002; Sofia *et al.* 2004), followed by *Gammaproteobacteria* (Sofia *et al.* 2004). *Archaea* were detected in all of the MBRs except in MBRs 5 and 7, but in low abundance.

Table 2.4: Semi-quantification of microbial population by FISH through epifluorescence microscopy

MBR plant	Archaea	*Proteobacteria* group			HGC (*Actino-bacteria*)	Ammonia oxidizing bacteria (AOB)	Nitrite oxidizing bacteria (NOB)	Polyphosphate accumulating organisms (PAO)			Glycogen accumulating organisms (GAO)
		Alpha	*Beta*	*Gamma*				Total PAOs	*Acc.* Type I[a]	*Acc.* Type II[a]	
MBR 1	•	•	•••	••	••	•	•	•	n.d.	n.d.	•
MBR 2	•	n.d.	••	••	•	•	n.d.	•	••••	••••	•
MBR 3	•	•	••••	••	•	•	•	•••	•••••	••	•
MBR 4	•	n.d.	••••	••	•	•	•	••	••••	••••	•
MBR 5	n.d.	n.d.	•••	•	•	n.d.	n.d.	••	n.d.	n.d.	n.d.
MBR 6	•	•	•••	••	•	n.d.	n.d.	••	n.d.	•	•
MBR 7	n.d.	•	•••	••	••	•	••	•	n.d	•	•
MBR 8	•	••	•••	••	•	•	•	••	•	•••••	••

[a]semi-quantification in respect to PAOmix; n.d.: not detected ; •: 1-10%; ••: 10-30%;•••: 30-50%; ••••: 50-70%; •••••: 70-85%; ••••••: 85-100%

2.3.2. Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) characterization

Ammonia oxidizing *Betaproteobacteria* were observed through FISH in all MBRs with a low relative abundance, except MBRs 5 and 6, where they were not detected (Table 2.4). Low or non-detection of AOB fluorescence signal was previously reported for MBR biomass (Luxmy *et al.* 2000; Witzig *et al.* 2002; Pala *et al.* 2008). AOBs were present in the form of small coccobacilli and always grouped in small-sized clusters, although in MBR 3 it was also possible to observe some big AOB cocci dispersed in the flocs. Manser *et al.* (2005) also reported the small size of AOB clusters in MBR, possibly related with the high shear forces imposed for membrane scouring. Regarding NOBs, none were detected in MBRs 2, 5 and 6 (Table 2.4). *Nitrobacter sp* was not identified in any MBR samples, which is consistent with previous findings (Wagner and Loy 2002; Kraume *et al.* 2005; Li *et al.* 2005; Manser *et al.* 2005), although Luxmy *et al.* (2000) reported bright signal detection with the NIT3 FISH probe. The only NOB cells detected in this study belonged to the genus *Nitrospira,* described in the literature as an active contributor to nitrite oxidation (Kraume *et al.* 2005; Li *et al.* 2005; Manser *et al.* 2005; Nielsen *et al.* 2009). *Nitrospira* generally presented cocci morphology, mostly aggregating in small clusters, though in the case of MBR 4, a rod morphology was also observed.

2.3.3. PAO and GAO characterization

Through applying a comprehensive set of probes targeting the PAO and GAO groups, it was demonstrated that PAOs were present in relatively higher abundance as compared to GAOs (Figure 2.1).

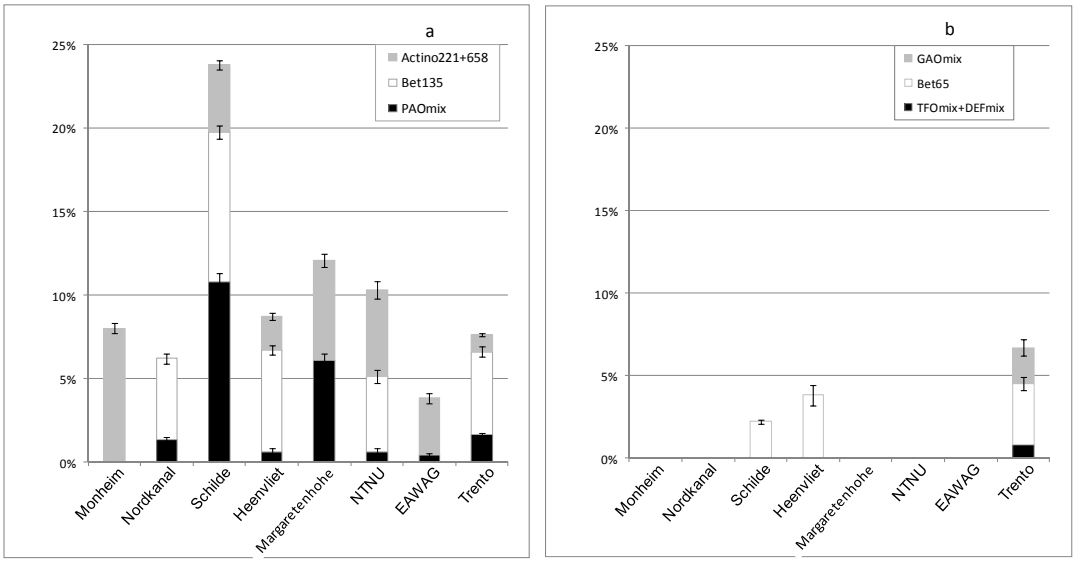

Figure 2.1: Quantitative FISH assessment of PAOs (a) and GAOs (b) in the MBR plants studied

Accumulibacter (PAOmix), presenting the commonly described morphologies (Carvalho *et al.* 2007; Oehmen *et al.* 2007), was absent in MBR 1, but was detected in the remaining systems (Figure 2.2).

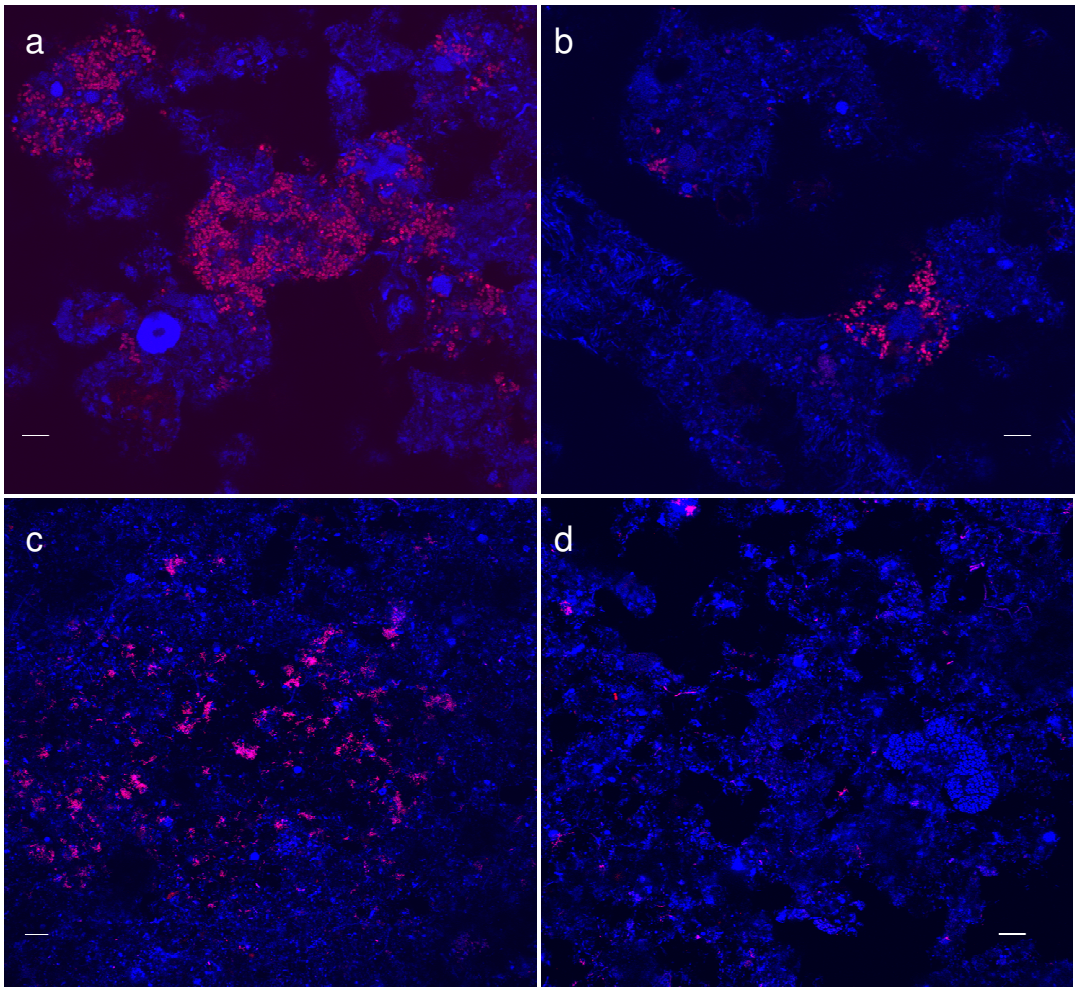

Figure 2.2: CLSM micrographs of biomass samples from (a) Schilde, (b and d) Heenvliet and (c) Monheim hybridized with probes for *Bacteria* (EUBmix, cells in blue) and for *Accumulibacter* (PAOmix, cells in magenta in a and b) or for *Tetrasphaera*-related *Actinobacteria* (Actino 221 and 658, cells in magenta in c and d). Bar = 10 µm

Nevertheless, *Accumulibacter* was only abundant in MBRs 3 and 5, where it accounted for 10.8% (SE_{mean} = 0.5%) and 6.1% (SE_{mean} = 0.4%) of the bacterial population, respectively. In the remaining MBRs, *Accumulibacter* was present in smaller abundance as compared to the *Tetrasphaera*-PAOs (Actino221+658) and/or the *Dechloromonas*-PAOs (Bet135). The *Tetrasphaera*-related

Actinobacteria were present within the range of 1-8% in the studied plants (Figures 2.1 and 2.2), except for MBR 2, where it was not detected. This group of PAOs was often the most abundant PAO identified, and in MBR 1 it was the only PAO present. The observed morphology of these cells was similar to that described by Kong *et al.* (2005), which were mainly short rods dispersed in the biomass, clusters of cocci in the shape of tetrads, and in lower abundance, other clusters of coccobacilli. The *Dechloromonas*-related PAO targeted by BET135 was detected at levels between 4-9% in the majority of the MBR plants except for MBRs 1, 5 and 7. Interestingly, this was the dominant PAO in MBR 4 (6.1%; SE_{mean}=0.3%), the full-scale EBPR plant, whereas it was not detected in the pilot-scale EBPR plant analyzed in this study (MBR 5). The morphologies of the BET135-targeted organisms included clusters of coccobacilli, as well as large cocci and rods that were more thinly dispersed in the biomass. These morphologies are in agreement with those described by Kong *et al.* (2007) for this group of putative PAOs. Additional FISH images of the different putative PAO groups can be viewed in Figure S2.1.

Overall, the GAOs were present in very low abundance in all the MBRs analyzed in this study (Figure 2.1), except for MBR 8. *Competibacter* was not found in any of the MBRs except for MBR 8, and in this plant it displayed a low relative abundance (2.2%; SE_{mean}=0.5%). Similar results were found for the probes targeting *Defluviicoccus vanus*-related *Alphaproteobacteria*: cluster 1 (TFOmix) was not detected and cluster 2 (DEFmix) was only observed in MBRs 3, 6 and 8, where small clusters of cocci were found in very low relative abundance (≤1%). Bet65-targeted cells were present in MBRs 3, 4 and 8 at levels ranging between 2 and 4%. These *Comamonadaceae*-related *Betaproteobacteria* were also detected in the other MBRs, except for MBR 5, although they were present in very low

abundance (<1%). A common morphology identified with Bet65 was short and medium size rods, as previously described (Kong *et al.* 2007), but clusters of coccobacilli were also observed. *Thioalkalivibrio*-related *Gammaproteobacteria* (Gam455) was not detected in any of the studied MBRs.

2.4. Discussion

All of the MBRs showed very high ammonia removal efficiencies, except for MBR 6 (Table 2.2), where no AOBs were detected by FISH analysis. MBR 6 was the only plant in this study with a very low sludge retention time (SRT), which likely justifies the absence of AOBs and NOBs, since these are slow growing autotrophic organisms that require a longer SRT in order to thrive. In MBR 5, no AOBs were detected, despite the broad coverage of the employed FISH probes, although NH_4-N was completely removed. Furthermore, no members of the *Archaea* domain were detected in this plant, suggesting the absence of archaeal ammonia oxidizers (AOA). The high NH_4-N consumption observed in this plant may have been partially consumed for the growth of heterotrophic biomass (MBR 5 had the highest biomass concentration). Additionally, unidentified AOBs and AOAs that are not covered by the employed FISH probes could be present in this MBR, as has been previously suggested in literature (Witzig *et al.* 2002; Chen and LaPara 2008).

Comparing the P-removal performance achieved in each plant (Table 2.2), MBRs 1 to 5 achieved low P effluent concentrations with a high total level of P-removal, particularly in MBRs 4 and 5, which were designed for EBPR. Between these two EBPR-MBRs, the highest P-removal was achieved in MBR 4, where *Accumulibacter* and *Tetrasphaera*-PAOs were present in low numbers (<2% each),

suggesting an active role of the putative *Dechloromonas*-related PAOs in biological P-removal (Figure 2.1). MBRs 7 and 8 were both pilot-scale plants and showed poorer P-removal as compared to the full-scale MBRs, even those not containing an anaerobic zone. Nevertheless, it should be pointed out that chemical precipitation was applied in MBRs 1 and 2, likely explaining the bulk of the P-removal achieved in these plants. Interestingly, MBR 3 did not contain an anaerobic zone, nor was chemical precipitation applied, but achieved the highest level of P-removal amongst non-EBPR plants. This result is in agreement with the FISH quantification values, which revealed that this plant contained the highest total putative PAO population, surprisingly even substantially higher than the EBPR plants (MBRs 4 and 5). The negligible biological P-removal achieved in MBRs 7 and 8 (not designed for EBPR) correlates well with the fact that the lowest quantity of PAOs was detected in MBR 7 (3.8%) and the highest quantity of GAOs was detected in MBR 8 (6.2%) (Figure 2.1). Moreover, the influent COD/P ratio was significantly higher in MBRs 7 and 8 (174 ± 29 mgCOD mg^{-1}P) than the other MBRs (39 ± 30 mgCOD mg^{-1}P), which also agrees well with the P-removal and microbial population results.

Not only was the total abundance of PAOs highest in a non-EBPR MBR, but the highest abundance of each individual group of putative PAOs (*Accumulibacter*, *Tetrasphaera*-PAOs and *Dechloromonas*-PAOs) was also observed in non-EBPR MBRs (Table 2.5).

Table 2.5: Abundance of EBPR-related microbial groups in this study and in full-scale WWTPs described in the literature

Plant Design	PAOs			GAOs			
	Accumulibacter **(PAOmix)**	***Dechloromonas*****-related** ***Betaproteobacteria*** **(Bet135)**	***Tetrasphaera*** **-related** ***Actinobacteria*** **(Actino221+658)**	***Competibacter*** **(GAOmix)**	***Comamonadaceae*****-related** ***Betaproteobacteria*** **(Bet65)**	***Defluviicoccus vanus*****-related** ***Alphaproteobacteria*** **(TFO_DFmix + DEFmix)**	***Thioalkalivibrio*****-related** ***Gammaproteobacteria*** **(Gam455)**
EBPR-MBR (present study)	<1-6%	<1-6%	2-6%	<1%	<1-4%	<1%	<1%
EBPR-CAS (literature)	7-12% [a] 6-16% [b] 1-19% [c] 4-18% [d]	<1-3% [e]	3-35% [c]	<1-12% [a] <1-3% [b] 10-31% [d]	<1-6% [e]	<1% [b]	<1-4% [e]
Non EBPR-MBR (present study)	<1 – 11%	<1 – 9%	<1-8%	<1 – 2%	<1 – 3%	<1 – 4%	<1%
Non EBPR-CAS (literature)	9-12%[d]	-	-	3-11%[d]	-	-	-

[a] Saunders *et al.* 2003; [b] Lopez-Vazquez *et al.* 2008; [c] Kong *et al.* 2005; [d] Wong *et al.* 2005; [e] Kong *et al.* 2007

In general, the PAO groups were within the range reported in literature, except for the *Dechloromonas*-PAOs, which presented higher abundances as compared to the EBPR plants studied in Kong *et al.* (2007), the only reported study to investigate these organisms (Table 2.5). Nevertheless, most previous studies have investigated the abundance of EBPR-related populations in EBPR plants; very few have presented results concerning the abundance of these organisms in non-EBPR plants, and no previous studies have investigated PAOs/GAOs in MBRs. Wong *et al.* (2005) performed the only other study comparing PAO/GAO abundance in EBPR-CAS vs non-EBPR-CAS plants. In their study, the abundance of *Accumulibacter* did not vary significantly among the different plants (9-12% in non-EBPR-CAS and 4-18% in EBPR-CAS). These results agree very well with our study, where the abundance of not only *Accumulibacter*, but also the other two putative PAO groups, was within a similar range for EBPR-MBR plants (total putative PAOs: 10 ± 2%) and non-EBPR-MBR plants (total putative PAOs: 10 ± 7%) (Figure 2.1).

These findings suggest that organisms that are considered to be putative PAOs can in fact thrive in systems (MBR or CAS) without anaerobic zones and grow to similar levels as EBPR plants. Their activity as PAOs is dependent on the operational conditions (e.g. presence of alternating anaerobic/aerobic conditions), but not necessarily their total numbers. In fact, putative PAOs can grow under a wide variety of environments (Peterson *et al.* 2008) and can behave as ordinary heterotrophs (Pijuan *et al.* 2006). To promote their activity as PAOs, the key is to impose appropriate operational conditions (normally involving an anaerobic zone), however, it is also possible that PAOs take advantage of anaerobic micro-niches occurring in non-EBPR plants. The high MLSS and EPS concentrations typical in MBRs (Judd 2008; Hall *et al.* 2010) might indeed facilitate the occurrence

of these micro-niches, which could justify the good biological P-removal observed in MBR 3. In such a situation, it is possible that a higher number of putative PAOs are required to achieve good P-removal performance as compared to a traditional EBPR-designed WWTP. For example, MBRs 4 and 5 were able to remove higher total quantities of P with lower numbers of putative PAOs (9-12%) as compared to MBR 3 (24%), for a similar total biomass concentration. It appears that the putative PAOs detected in MBRs 4 and 5 were behaving as PAOs much more efficiently as compared to MBR 3, a non-EBPR MBR system.

The apparent adaptability and metabolic flexibility of putative PAOs in activated sludge systems with or without a well defined anaerobic phase is in agreement with the metagenomic analysis of *Accumulibacter* (Martin *et al.* 2006). This study showed several metabolic capabilities that could be expressed according to the adaptation required to the surrounding environment, such as the presence of high affinity P transporters to scavenge P when present at low concentrations, and a complete set of genes to perform nitrogen fixation, which would enable survival in e.g. nutrient limited-habitats. Furthermore, MBRs are typically operated with high biomass concentrations, resulting in low F/M ratios (Table 2.2), which in turn leads to a limited ATP supply to the biomass (Low and Chase 1999; Witzig *et al.* 2002; Monclus *et al.* 2010). Thus, microorganisms capable of alternate means of satisfying their maintenance energy requirements are positively selected in MBRs. In this way, the numbers of PAOs in non-EBPR-MBRs could be explained by both their metabolic flexibility and their ability to accumulate an ATP source (i.e. polyphosphate granules) that can be used to fulfill their energetic requirements in substrate-limited conditions.

Wong *et al.* (2005) found that in EBPR-CAS plants, *Competibacter* reached values three times higher than in non-EBPR-CAS. In this study, GAOs were often present in low abundance (Table 2.5), thus, no trend could be established with respect to the impact of operational conditions typical of MBR technology on GAO selection. It is still unknown if GAOs are as adaptable to non anaerobic/aerobic environments as PAOs appear to be. The operational conditions that lead to the proliferation of PAOs over GAOs in MBRs and the promotion of biological phosphorus removal in these systems are topics of interest for future research.

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CHAPTER 3

Step-by-step strategy for protein enrichment and proteome characterization of extracellular polymeric substances in wastewater treatment systems

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Silva AF. was involved in all the experimental work presented in this chapter, except for the operation of the lab-scale membrane bioreactor and biomass sampling; The protein identification by MALDI-TOF/TOF and MASCOT search was performed by Renata Soares and Ana Varela Coelho.

CONTENTS

Abstract

Extracellular polymeric substances (EPS) are key in biomass aggregation and settleability in wastewater treatment systems. In membrane bioreactors (MBR), EPS is an important factor as it is considered to be largely responsible for membrane fouling. Proteins were shown to be the major component of EPS produced by activated sludge and to be correlated with the properties of the sludge, like settling, hydrophobicity and cell aggregation. Previous EPS proteomic studies of activated sludge revealed several problems, like the interference of other EPS molecules in protein analysis. In this study, a successful strategy was outlined to identify the proteins from soluble and bound EPS extracted from activated sludge of a lab-scale MBR. EPS samples were first subjected to pre-concentration through lyophilisation, centrifugal ultrafiltration or concentration with a dialysis membrane coated by a highly absorbent powder of polyacrylate-polyalcohol, preceded or not by a dialysis step. The highest protein concentration factors were achieved with the highly absorbent powder method without previous dialysis step. Four protein precipitation methods were then tested: acetone, trichloroacetic acid (TCA), perchloric acid and a commercial kit. Protein profiles were compared in 4-12% SDS-PAGE gels. Both acetone and TCA should be applied for the highest coverage for soluble EPS proteins, whereas TCA was the best method for bound EPS proteins. All visible bands of selected profiles were subjected to mass spectrometry analysis. A high number of proteins (25–32 for soluble EPS and 17 for bound EPS) were identified. As a conclusion of this study, a workflow is proposed for successful proteome characterisation of soluble and bound EPS from activated sludge samples.

3.1. Introduction

Biological systems are the most commonly employed processes for wastewater treatment worldwide. In these processes, the microorganisms are withheld in the system thanks to their capacity of forming bioaggregates (flocs and granules, with higher settling velocity than individual cells), or biofilms. Cell aggregation is possible due to the complex network established through extracellular polymeric substances (EPS), where cells are embedded. Besides facilitating cell aggregation, EPS act as a protective matrix from external aggressions (dewatering, toxic substances, high temperature and pH) and provide means for easy access to nutrients, for genetic information exchange and for extracellular enzymatic reactions (Wingender *et al.* 1999; Flemming and Wingender 2010; Sheng *et al.* 2010).

Despite all the important functions mediated by EPS, in some situations EPS can also be cumbersome to engineered systems. An excessive amount of EPS can reduce the mass transfer processes occurring in wastewater treatment plants (Sheng *et al.* 2010). Moreover, the permeability of membrane bioreactors (MBR) is severely affected by the accumulation of EPS at the membrane surface and pores, which results in increased operation and capital costs (Drews 2010). Although the correlation between soluble EPS and membrane fouling is supported by several studies, it is still not clear which functional groups within the wide variety of molecules comprising EPS have a more prominent role in fouling (Drews 2010). In sum, either for promoting cell aggregates retention or for interfering with process performance, it is well established in literature that EPS have an important effect in wastewater treatment systems. However, little is known about

the molecular composition of EPS and the role of specific macromolecules in the matrix.

EPS are a complex mixture of biopolymers with high molecular mass which include mainly proteins, polysaccharides and humic acids. Extracellular DNA and other polymeric compounds such as glycoproteins and glycolipids are also present although in smaller amounts (Flemming *et al.* 2007). These molecules are produced and excreted by microorganisms, or released as a result of cell lysis, and also comprise other organic molecules present in the wastewater that adsorb to the flocs (Wingender *et al.* 1999).

Many EPS studies in wastewater treatment processes have focused on the analysis of proteins and polysaccharides as a whole, in order to investigate which fraction had higher relevance in process efficiency (Wang *et al.* 2010; Bala Subramanian *et al.* 2010). Although polysaccharides have been reported as the major macromolecule type in EPS from pure cultures, in activated sludge and other environmental samples proteins have been described as the most abundant EPS (Flemming and Wingender 2010; Badireddy *et al.* 2010; Frolund *et al.* 1996; D'Abzac *et al.* 2010; Liu and Fang 2002; Park *et al.* 2008). Biofilm studies showed that EPS proteins are involved in the bacterial adhesion to surfaces and in bridging between cells, and thus have a role in biofilm cohesion. Moreover, proteins confer a protective barrier to the cells and are responsible by the enzymatic activity for the exogenous digestion of macromolecules. This wide range of functions reflects the high diversity of proteins found in EPS. Therefore, protein quantification may be insufficient to understand their role in EPS. The identification of the extracellular proteins retrieved in wastewater treatment systems may indicate their function in EPS, as well as their effect in the systems' performance.

Ultimately, protein identification can lead into the identification of microbial community members that contributed to that effect.

Previous studies have attempted the identification of EPS proteins in wastewater treatment systems (Park *et al.* 2008; Higgins and Novak 1997; Denecke 2006; Kuhn *et al.* 2007). Higgins and Novak (1997) targeted extracellular proteins to study their relationship with the settling and dewatering properties of activated sludge. Although they observed a direct correlation between the bound EPS protein content and the sludge settling properties, the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation only enabled the identification of a single protein. Denecke *et al.* (2006) extracted proteins from crude sludge extract but the corresponding SDS-PAGE gel presented very strong smears masking the bands. Park *et al.* (2008) investigated the extracellular proteins in activated sludge flocs using different EPS extraction methods, but were only able to identify few proteins, half of them of human origin, thus not produced by the activated sludge biomass. The complexity of the EPS matrix likely hinders protein purification from other macromolecules. Namely, the presence of humic acids is known to interfere with protein visualisation in SDS-PAGE (Denecke 2006). This could explain the low number of EPS proteins previously identified in wastewater treatment systems.

The aim of this study was to define a step-by-step strategy to concentrate, enrich and identify the extracellular proteins present in the soluble and bound EPS extracted from activated sludge. Previously described procedures were compared and a novel multiple-step strategy was developed with the purpose of overcoming difficulties encountered in earlier studies for protein identification from EPS.

3.2. Materials and methods

3.2.1. Lab-scale membrane bioreactor

The activated sludge samples were collected from a laboratory scale membrane bioreactor (MBR) comprising a 13 L anoxic tank and a 17 L aerobic tank, where a module of polysulphone hollow fibre membranes (Polymem, Tolouse, France), with an area of 0.1 m^2 and a pore diameter of 0.2 µm, was submerged. The MBR was operated at a sludge retention time of 60 days, a hydraulic retention time of 1 day and an average flux of 12 ± 1.5 $Lm^{-2}h^{-1}$. Three samples (sample 1, sample 2 and sample 3) were collected from the aerobic tank throughout a period of 4 months of operation. The reactor was operated with an average mixed liquor suspended solids (MLSS) of 7 (±1.9) gL^{-1} during the sampling period, an aeration rate of 35 L min^{-1}, and a pH of 7.0 ± 0.1. The feed was composed of domestic wastewater collected at the Mutela wastewater treatment plant (Almada, Portugal) supplemented with sodium acetate to a total chemical oxygen demand of 476-860 mg L^{-1}**.**

3.2.2. EPS extraction and quantification

EPS was extracted immediately after sample collection. EPS can be divided into two fractions: soluble and bound EPS, in which soluble EPS is the set of biopolymers and colloids dissolved in solution, whereas bound EPS is the set of biopolymers that are closely bound to the microbial cells (Wingender *et al.* 1999; Sheng *et al.* 2010).

Soluble EPS was obtained by collecting the supernatant after centrifuging 400 mL of activated sludge (12000 g, 15 min, 4 °C). The bound EPS was extracted through incubation with a cation exchange resin (CER) (Dowex® MARATHON®, 20-50 mesh, Sigma Aldrich, St Louis, USA) according to Frolund *et al.* (1996) with minor modifications. In brief, 75 mg of the Na^+ form of CER per g of volatile suspended solids (VSS) was added to the biomass pellet (obtained after removal of the soluble EPS) resuspended in 400 mL EPS extraction buffer (2 mM Na_3PO_4, 4 mM $NaH_2PO_4 \cdot 12H_2O$, 9 mM NaCl, 1 mM KCl, pH 7), and stirred at 600 rpm for 2 h at 4 °C. Next, the sample was centrifuged at the same conditions described above, recovering the bound EPS fraction. Both EPS fractions were preserved at -20 °C until further analysis.

Total protein content was determined by the Lowry method (Frolund *et al.* 1996), using bovine serum albumin (BSA, Merck KGaA, Germany) as standard. Total polysaccharide content was determined by the Dubois protocol (Dubois *et al.* 1956), and calibration was performed with glucose (Sigma chemical Co, St Louis, USA). Measurements were performed in an Ultrospec 2100 pro spectrophotometer (Biochrom Ltd, Cambridge, England) and all samples were measured in duplicate.

3.2.3. Concentration of EPS proteins

Triplicate 25 mL aliquots of the soluble and bound EPS samples were placed in dialysis tubings of regenerated cellulose with a 10 kDa cut-off (SnakeSkin dialysis tubing, Thermo Scientific, Rockford, USA). The closed tubings were coated with a powder of polyacrylate-polyalcohol (PP) absorbent gel (Spectra/Gel®

Absorbent, SpectrumLabs, Rancho Dominguez, CA, USA), which is highly absorbent and promotes the diffusion of water and smaller molecules through the dialysis membrane. This step was carried out at 4 °C overnight. The powder was then carefully removed and samples were recovered. A second concentration process was tested for two of the samples (soluble and bound EPS of sample 2), consisting of the use of ultrafiltration centrifugal devices where a membrane of regenerated cellulose is welded within a conical device (Pierce Protein concentrators 9 k MWCO, Thermo Scientific, Rockford, USA). In this case, after rinsing the membrane, 10 mL were centrifuged at 6000 g in a fixed angle rotor (SIGMA4K15, SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany) at 22 °C and concentrated samples were recovered. Another concentration method applied was lyophilisation: 25 mL aliquots of each sample were frozen and placed overnight in a lyophiliser (Modulyo 4K Freeze Dryer, Edwards High Vacuum International, West Sussex, UK) to dehydrate the samples. The last concentration method compared was a two-step process. First, 25 mL aliquots were placed in the same type of dialysis tubings as described above (regenerated cellulose with a 10 kDa cut-off) and dialysed overnight against 2.5 L of buffer containing 2 mM of Na_3PO_4, 4mM of NaH_2PO_4 $12H_2O$, 9 mM of NaCl and 1 mM of KCl. Next, the samples were concentrated with PP absorbent gel, as previously described. The protein and polysaccharide content of the concentrated samples were measured as described above.

3.2.4. Protein precipitation

Four different precipitation protocols were tested in duplicate for each sample: acetone 4x, trichloroacetic acid (TCA) 13%, perchloric acid 5% and

ProteoPrep® Protein precipitation kit. For the acetone precipitation method, 1 volume of sample was added to 4 volumes of cold acetone (J.T. Backer, Deventer, The Netherlands), mixed thoroughly and incubated overnight at -20 °C. After centrifugation (16000 g, 15 min, 4 °C), the supernatant was rejected and the pellet was air dried. For TCA precipitation, the acid (Panreac, Barcelona, Spain) was added to the samples to a final concentration of 13%, mixed thoroughly and incubated overnight at 4 °C. This method is particularly indicated for proteins in low concentration (Evans *et al.* 2009). Next, the mixture was centrifuged (16000 g, 15 min, 4 °C) and the supernatant discarded. For precipitation with perchloric acid, the acid (Merck KGaA, Darmstadt, Germany) was added to the samples to a final concentration of 5%, vortexed for 30 - 60 seconds at room temperature and centrifuged (16000 g, 15 min, 4 °C). The supernatant was rejected. The precipitation with the ProteoPrep® kit followed the manufacturer's instructions (Sigma, St Louis, USA). All pellets were resuspended with minimum volume of NuPAGE® LDS sample buffer (4x) (Invitrogen, Carlsbad, CA, USA) required to dissolve it: 5 µL for soluble EPS and 9 µL for bound EPS. NuPAGE® LDS is a commercial buffer used to prepare samples for gel electrophoresis in denaturating conditions.

3.2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the NuPage® electrophoresis system instructions (Invitrogen, Carlsbad, CA, USA). Protein separation was performed in precast NuPAGE Novex® Bis-Tris gels (1 mm thick and 8 mm long) with a polyacrylamide concentration gradient of 4-12%. 1.5 µL of NuPAGE® Reducing

Agent were added to each resuspended sample. The mixture was heated to 70 °C and the whole volume was immediately applied onto the gel. The protein standard applied was the Precision Plus All Blue (Bio-Rad Laboratories Inc., CA, USA). Electrophoresis was run in 2-(N-morpholino) ethanesulfonic acid (MES) 1x plus 0.25 % (v/v) NuPAGE® antioxidant at a constant voltage of 200 volts for 30 min. Gels were stained with SimplyBlue™ SafeStain (Coomassie G-250) (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

The correlation between protein fingerprints from different precipitation protocols was statistically analysed. Similarities between the gel fingerprints were calculated with Bionumerics 6.0 software (Applied Maths, Belgium), clustering the different profiles by means of the Pearson product-moment correlation coefficient and unweighted pair group method of arithmetic averages (UPGMA). The correlation coefficient was used to calculate the two tailed *p*-value. Differences between profiles were considered to be statistically significant with a confidence interval of 95% when the p-value was lower than the significance value (α) of 0.05.

3.2.6. Protein identification

The gel bands of interest were excised and in-gel digested with trypsin as described (Santos *et al.* 2009). The tryptic digests were desalted and concentrated through chromatographic microcolumns using GELoadder tips containing POROS R2 (20 µm bead size, Applied Biosystems, USA) and the bound peptides were eluted with 0.6 µL of 5 mg mL^{-1} α-ciano-4-hydroxycinnaminic acid (Sigma-Aldrich,

USA) in 50% (v/v) acetonitrile (Merck, KGaA, Germany) with 5% (v/v) formic acid (Fluka Sigma-Aldrich, Germany).

Mass spectrometry (MS) analysis was performed in a 4800 Plus matrix-assisted laser desorption/ionization - tandem time-of-flight coupled to a mass spectrometer (MALDI-ToF/ToF MS) (Applied Biosystems, USA) in reflectron positive MS and MS/MS modes, excluding contaminating peaks from trypsin autodigestion. Each reflectron MS spectrum was collected in a result-independent acquisition mode, typically using 500 laser shots per spectrum and a fixed laser intensity of 3300 V. Fifteen of the strongest precursors were selected for MS/MS, with the strongest precursors being fragmented first. Two thousand laser shots were collected for each MS/MS spectrum using collision induced dissociation (CID) assisted with air, with collision energy of 1 kV and a gas pressure of 1×10^{6} torr.

Protein identification was performed using the algorithm MOWSE® from MASCOT (version 2.2; Matrix Science, USA) in combined search mode using MS and MS/MS data and two general protein sequence databases, SwissProt (release 2011_01; 524,420 entries; European Bioinformatics Institute) and NCBInr (version 06082010, 11,592,253 entries). The search criteria used for the protein identification searches were 50 ppm tolerance for the precursor ion and 0.3 Da tolerance for the MS/MS fragments. Other search parameters were set as follows: enzyme, trypsin; Cys alkylation, iodoacetamide; Met oxidation as fixed and variable modifications, respectively. No taxonomy restrictions were defined.

Protein identification was accepted when the protein score was above the MASCOT score (83 for NCBInr and 70 for SwissProt) and at least one peptide fragmented with 95% confidence for both databases.

3.3. Results

3.3.1. EPS extraction and protein concentration

The major challenge in the proteome characterisation of EPS samples is the difficulty to obtain visible, well resolved and contaminant-free protein bands in electrophoresis gels that enable further identification. This is probably due to the low protein concentration in this type of samples and to the co-extraction of other EPS molecules (Denecke 2006; Seviour *et al.* 2010). Therefore the first step in this study consisted of assessing these two factors.

3.3.1.1. Protein and polysaccharide quantification in activated sludge samples

The cation exchange resin EPS extraction method (Frolund *et al.* 1996) was selected based on previous comparative studies, which showed that this method had the highest efficiency of protein extraction in detriment of humic acids, while inducing very low cell lysis, and as such avoiding contamination with intracellular proteins (Wingender *et al.* 1999; Frolund *et al.* 1996; D'Abzac *et al.* 2010; Liu and Fang 2002; Comte *et al.* 2006). The concentrations of EPS proteins and polysaccharides in the activated sludge samples before concentration are given in Table 3.1. Although in the same order of magnitude, protein concentrations were higher than polysaccharides, particularly in the bound EPS. The obtained protein concentrations varied between 1 – 2 μg protein mg VSS^{-1} for soluble EPS and 17 – 26 μg protein mg VSS^{-1} for bound EPS, which was within the range of the protein extraction results of other studies performed with activated sludge (Liu and Fang 2002; Sheng *et al.* 2005).

Table 3.1: Protein and polysaccharide concentrations (average ± standard deviation) in soluble and bound EPS samples before and after concentration through the use of a dialysis tubing coated with PP absorbent gel and ultrafiltration centrifugal devices

	Original sample		After sample concentration		
			PP absorbent gel		Ultrafiltration centrifugal devices
	Protein ($\mu g\ mgVSS^{-1}$)	Polysaccharide ($\mu g\ mgVSS^{-1}$)	Protein ($\mu g\ mgVSS^{-1}$)	Polysaccharide ($\mu g\ mgVSS^{-1}$)	Protein ($\mu g\ mgVSS^{-1}$)
Soluble EPS sample 1	1.2±0.2	1.2±0.8	7.6±3.0	2.5±0.7	-
Soluble EPS sample 2	1.8±0.5	0.8±0.7	6.4±1.2	1.7±0.4	0.4±0.0
Bound EPS sample 2	17.2±1.1	10.1±0.4	21.5±1.1	8.1±0.0	9.0±0.1
Bound EPS sample 3	26.2±0.4	4.6±0.8	46.1±3.3	13.0±1.1	-

Direct precipitation to purify and concentrate proteins, before separating them through electrophoresis, is commonly used in intracellular metaproteomic studies (Benndorf *et al.* 2009; Wilmes and Bond 2006). This approach was also tested in this study, however, direct application of precipitation to EPS samples required large volumes of solvent, since a large initial sample was necessary to obtain a final measurable amount of protein. This process was inefficient to precipitate both soluble and bound EPS samples in this study, since the amount of protein retrieved, although detectable by the Lowry method, was not visible in the SDS-PAGE gels. Therefore, a pre-concentration step was necessary.

3.3.1.2. Selection of a protein concentration process

Several methods were compared to pre-concentrate the EPS samples: namely lyophilisation, centrifugal ultrafiltration and application of PP absorbent gel on a dialysis tubing containing the sample, preceded or not by dialysis. Conventional dialysis against a buffer, while effective in reducing the content of contaminants with a molecular weight below the membrane cut-off, did not increase the efficiency of protein precipitation, still requiring further concentration steps to obtain detectable protein amounts. Sample lyophilisation resulted in an insoluble powder, possibly indicating that structural modifications took place in the EPS macromolecules during the process, as suggested by Buffle and Leppard (1995). Therefore, sample concentration through the use of PP absorbent gel or ultrafiltration centrifugal devices were selected as the most promising methods for further comparison, enabling protein recovery for subsequent separation steps with simultaneous reduction in the content of contaminants.

When comparing these two methods, higher protein concentrations were obtained with the PP absorbent gel method than with the centrifugal ultrafiltration, in the three replicates of each sample, with an average concentration factor of 6x for soluble EPS and 2x for bound EPS (Table 3.1). Although the other macromolecules were also concentrated in the absorption process, the polysaccharide concentration factor was smaller (2x) in soluble EPS, and in one of the samples, the final concentration was even lower than the original value, which suggests that in this fraction a significant part of the polysaccharides had a size bellow the dialysis membrane cut-off and were washed out during the process. Furthermore, the bound EPS samples, which were initially translucid, became brownish after concentration through application of the PP

absorbent gel, which may indicate that the humic acids were abundant in this fraction and were also concentrated.

When applying the ultrafiltration centrifugal devices, which had the same cut-off as the dialysis tubing used for the PP absorbent technique, protein loss was observed for both EPS fractions (soluble EPS concentration factor of 0.2x; bound EPS concentration factor of 0.5x – Table 3.1). The failure in concentrating the proteins, and the presence of an insoluble brown deposit on the ultrafiltration membranes after centrifugation may indicate that other molecules from the complex EPS matrix, may be interacting with the membrane, although these type of membranes are designed for minimizing protein polarisation and adsorption to its surface (according to the manufacturer's instructions).

In view of these results, the subsequent steps were only performed with the samples concentrated using the PP absorbent gel method.

3.3.2. Protein separation in SDS-PAGE gels

The application of the samples concentrated using the PP absorbent gel method in SDS-PAGE gels resulted in a very low number of faint and diffuse protein bands, difficult to excise for further processing (Figure 3.1A, lanes 8 and 15). Furthermore, only part of the EPS components that could interfere in obtaining well contrasted bands was removed during the concentration procedure with the PP absorbent gel. Therefore, it was necessary to further concentrate and purify the proteins to obtain a defined gel fingerprint, with higher number of proteins detected.

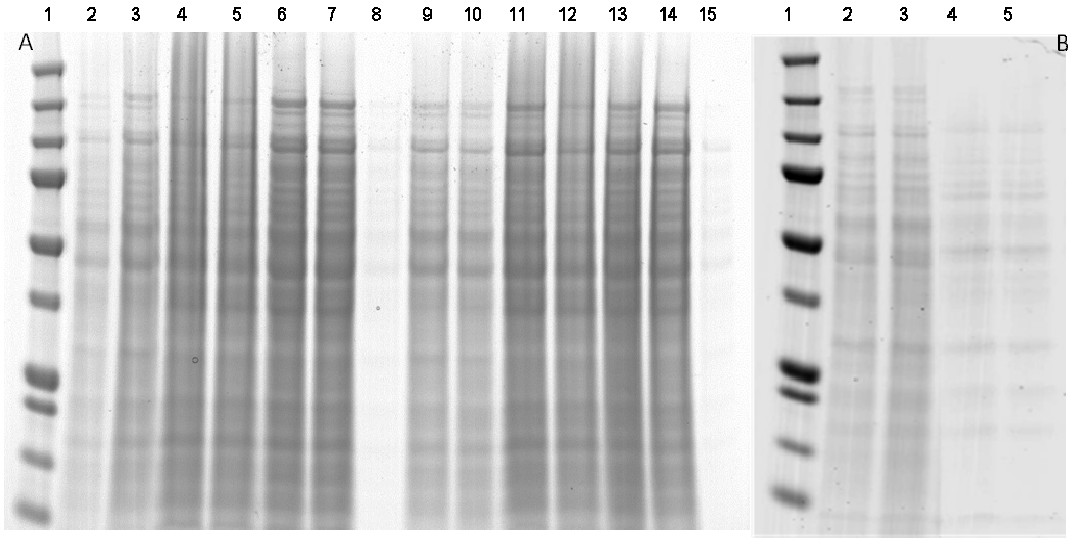

Figure 3.1: SDS-PAGE of soluble EPS samples after protein concentration and precipitation: (A) Soluble EPS sample 1 concentrated with Spectra Absorbent method, replicate 1 (wells 2 to 8) and replicate 2 (wells 9 to 15), (B) Soluble EPS sample 2 concentrated, replicate 1. Gel A) lanes 2, 3, 9 and 10: acetone-precipitated; lanes 4, 5, 11 and 12: TCA-precipitated; lanes 6, 7, 13 and 14: perchloric acid-precipitated; lanes 8 and 15: direct application of concentrated samples without precipitation. Gel B) lanes 2 and 3: acetone-precipitated; and lanes 4 and 5: precipitated with the ProteoPrep® kit. Lanes 1 of both gels: molecular weight marker

3.3.2.1. Protein precipitation

Due to the complex nature of the samples, four different precipitation protocols were compared: acetone 4x, TCA 13%, perchloric acid 5% and the commercial kit ProteoPrep®.

Precipitation with acetone or TCA are commonly applied methodologies to purify proteins (Evans *et al.* 2009). The perchloric acid method was previously applied to precipitate proteins from different matrix (Seviour *et al.* 2010; Ghasemi *et al.* 2007). The ProteoPrep® kit is indicated for proteins present in

aqueous samples as, according to the manufacturer, the combination of TCA with deoxycolate (DOC) enhances high protein yields due to the co-precipitation of proteins with DOC in acidic conditions.

The acetone, TCA and perchloric acid methods successfully resulted in pellet formation for all the tested samples. Pellets obtained from soluble EPS samples were completely soluble in NuPAGE® LDS sample buffer. For bound EPS samples, the TCA and perchloric acid methods yielded pellets which were much more difficult to solubilise than the other two methods. Adding this result to the fact that the pellets obtained were brownish, it may indicate a co-precipitation of other EPS components not so easily dissolved in a buffer designed to dissolve proteins. The pellets obtained using the ProteoPrep® kit were larger than the ones obtained with other methods, which was expected due to the co-precipitation of the DOC with the proteins. However, these pellets were not compact enough, which resulted in unselective loss of proteins when removing the supernatant. In order to assess which precipitation method was able to retain the highest protein diversity, the proteins obtained with each method were resolved through SDS-PAGE.

3.3.2.2. Protein separation by gel electrophoresis

Preliminary experiments carried out with concentrated and precipitated EPS samples on 12% SDS-PAGE gels resulted in very limited protein separation (maximum three poorly defined bands, results not shown). Multiple proteins likely co-eluted in each band, as suggested by subsequent MALDI-ToF/ToF MS analysis, which was unsuccessful due to unresolved mixture of peaks. A clearly better band

pattern resolution was achieved when using 4-12% gradient polyacrylamide gels, which were thereafter used in the rest of the study.

A common feature in the SDS-PAGE gels obtained for all the precipitation methods, both for soluble and bound EPS proteins, was a smear observed throughout the lanes (Figures 3.1 and 3.2). TCA and perchloric acid protocols led to gels with strongest smear. This smear was probably due to the co-precipitation and migration of humic acids, as humic acids are also stained with Coomassie (Denecke 2006; Taylor and Williams 2010). However, the smear did not prevent band detection and protein identification through mass spectrometry (see below).

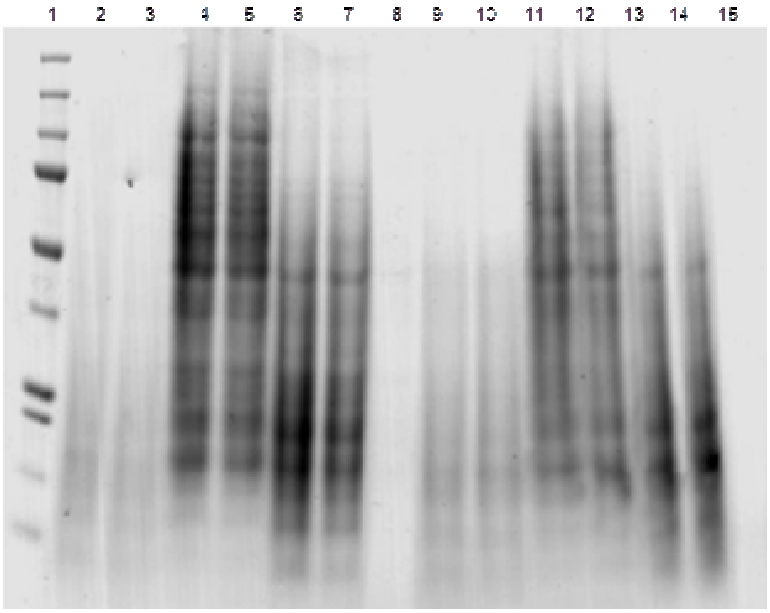

Figure 3.2: SDS-PAGE of bound EPS sample 3 replicate 1 (lanes 2 to 8) and 2 (lanes 9 to 15) after protein concentration and precipitation. Lane 1: Molecular weight marker; lanes 2, 3, 9 and 10: acetone-precipitated; lanes 4,5,11 and 12: TCA-precipitated; lanes 6, 7, 13 and 14: perchloric acid-precipitated and lanes 8 and 15 correspond to the direct application of concentrated samples, without precipitation

Precipitation of concentrated soluble EPS proteins with organic solvents or acids (acetone, TCA and perchloric acid) resulted in a broad protein range of molecular masses (10 – 200 kDa) with complex patterns of about 25 defined bands each (Figure 3.1A and B). The same samples precipitated with the ProteoPrep® kit resulted in smear-free fingerprints but with fewer bands than those obtained with the other protocols, an expected outcome due to the pellet loss observed previously. All bands obtained with the kit matched bands observed with the acetone precipitation protocol, but, although with the commercial kit a cleaner profile was attained, it was only able to detect the most abundant proteins (Figure 3.1B). Overall, the acetone protocol gave the highest number of bands with lowest level of smear, resulting in the most clear complete pattern.

For soluble EPS proteins from sample 1, the patterns of bands obtained with TCA and perchloric acid precipitation were closer between them (*p*-value 0.22) than the one obtained with the acetone method (*p*-value <0.03), but for soluble EPS proteins from sample 2, the protein fingerprint was similar for all the precipitation protocols (*p*-value 0.25). This may indicate that the proteins in the extracellular extract of the two samples behave distinctly with an acid or a ketone precipitation protocol. All precipitation protocols were reproducible (*p*-value of replicates was: 0.24 for acetone; 0.4 for TCA; 0.23 for perchloric acid; 0.26 for ProteoPrep kit).

Precipitation of proteins from bound EPS with acetone resulted in gel fingerprints with unclear bands (Figure 3.2). The same result was obtained with the commercial kit ProteoPrep® (results not shown). With TCA and perchloric acid methods, despite the intense smear, it was possible to observe a substantial number of defined bands, 20 for the TCA fingerprint and 15 for the perchloric acid

protocol. A hypothesis to explain this smear is the co-precipitation of other EPS molecules, like humic acids (which would also explain the brownish colour of the pellets), that were not well solubilised with the sample buffer, being dragged throughout the gel. All precipitation protocols showed reproducible gel patterns for bound EPS (*p*-value of replicates was: 0.52 for acetone; 0.26 for TCA; 0.29 for perchloric acid; 0.26).

3.3.3. Protein identification by MALDI ToF-ToF/MS

In order to confirm that the bands obtained in SDS-PAGE gel after sample concentration and precipitation corresponded to well-resolved proteins, representative bands of each protocol of interest were selected for protein identification. Thus, all visible bands from the following SDS-PAGE lanes were excised, trypsin-digested and analysed through MALDI-ToF-ToF/MS: soluble EPS of sample 2 (acetone- and TCA- precipitated), soluble EPS of sample 1 (perchloric acid- precipitated), and bound EPS of sample 3 (TCA-precipitated).

Table 3.2 shows the results of protein identification. All protocols enabled the separation and identification of a high number of EPS proteins. Several gel bands resulted in the identification of more than one protein (an average of 1.1 proteins with successful identification per band). This means that different proteins had the same migratory behaviour in the SDS-PAGE gel. Comparing the results obtained from acetone and TCA precipitation for soluble EPS of sample 2, both protocols were similarly successful in respect to total number of identified proteins. However, each protocol identified some exclusive proteins: six for acetone and seven for TCA, making both protocols complementary.

Table 3.2: Protein identification results from selected bands subjected to trypsin digestion and MALDI-MS analysis.

EPS fraction	Sample	Precipitation protocol	No. of bands trypsin digested	No. of proteins identified
Soluble	**1**	Perchloric acid	28	33
Soluble	**2**	Acetone	26	27
Soluble	**2**	TCA	26	30
Bound	**3**	TCA	20	18

All the proteins identified were of bacterial origin and very diverse in function and cellular origin, according to Protein Knowledgebase UniProtKB/Swiss-Prot database search (Figure 3.3). In terms of the soluble EPS fraction, the majority of proteins were reported as originated in the cytosol/cytoplasm subcellular compartment (Figure 3.3A). Approximately half of the identified proteins were structural proteins (part of the structure of the cell or cell components), followed by proteins functioning as elongation factors (half of them located in the membrane), transporters and transferases (Figure 3.3B). For bound EPS, the identified proteins were also mainly originated in the cytosol/cytoplasm subcellular compartment (Figure 3.3C). Concerning their molecular function, the majority were structural proteins, followed by elongation factors (Figure 3.3D).

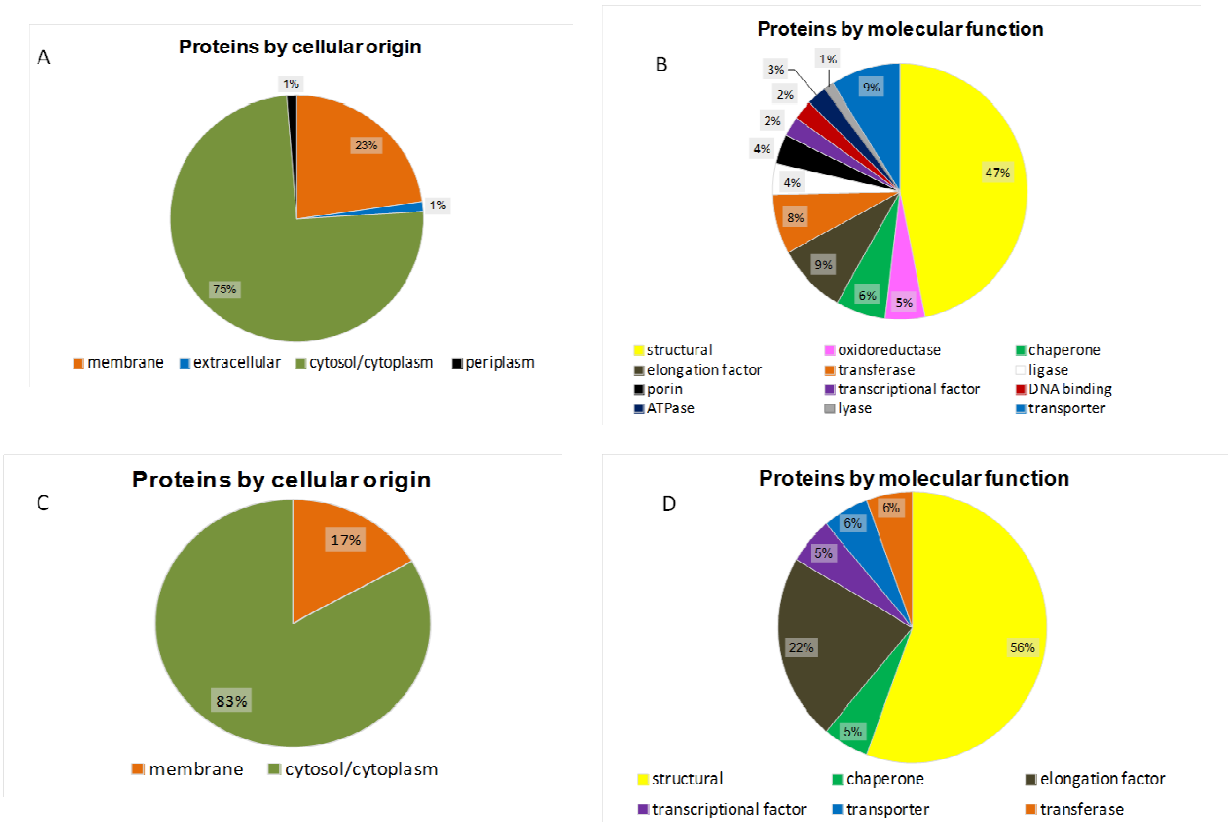

Figure 3.3: Protein identification results for soluble EPS sample 2, by (A) cellular origin and by (B) molecular function, and for bound EPS sample 3 by (C) cellular origin and (D) molecular function, according to Protein Knowledgebase UniProtKB/Swiss-Prot database

3.4. Discussion

The main objective of this study was to define an approach to concentrate, purify and identify the proteins that compose the EPS of biological wastewater treatment systems. EPS were extracted in two fractions that were concentrated and partially cleaned from EPS components below a molecular weight of 10 kDa. These concentrated fractions were subjected to selected precipitation protocols. Proteins were separated by SDS-PAGE and selected protein profiles were excised and trypsin-digested for protein identification by mass spectrometry. Overall, the extracellular protein content in the MBR soluble and bound EPS samples was in higher concentrations than the polysaccharides.

In this initial step in protein recovery, the influence of the extraction method in the yield of EPS components extracted is well known. The cation exchange resin method was chosen because it was demonstrated in previous studies that it presents a good compromise between protein yield and cell integrity, it has high humic acids rejection, which is relevant for a clearer resolution of the SDS-PAGE gels, and does not interfere with the subsequent steps of analysis (Frolund *et al.* 1996; D'Abzac *et al.* 2010; Liu and Fang 2002; Comte *et al.* 2006). Indeed, other EPS extraction methods, e.g. using EDTA or detergents, may leave residual amounts of chemicals that can interfere with sample ionisation, adduct formation and ion-source fouling of the MALDI-MS used for protein identification (Evans *et al.* 2009).

Despite being the major EPS component, proteins were still found in too low concentrations in soluble and bound EPS samples to ensure clear band detection in subsequent SDS-PAGE analysis, good resolution and broad EPS protein coverage. In fact, protein purification applying widely-used precipitation with

solvents directly on extracted samples revealed to be inefficient, with only very few and faint bands being retrieved. Increasing the initial sample volume was not a feasible solution for this lab-scale analytical approach, since it would require unpractical and costly amounts of solvents (some of which quite toxic).

Sample pre-concentration proved to substantially enhance the efficiency of protein purification through precipitation. From the tested techniques, absorption of part of the matrix (mostly water, salts and low molecular-weight solutes) with the PP absorbent gel showed to be an efficient single-step method, with minimal protein loss. This concentration method had the additional benefit of reducing the content in EPS molecules below 10 kDa, which, in the case of soluble EPS, resulted in a clear enrichment of protein content in detriment of the polysaccharide fraction, suggesting that a large portion of the polysaccharides in soluble EPS were below the dialysis tubing cut-off. However, the use of the PP absorbent gel as the sole concentration/purification step was found to be insufficient to clean samples from the rest of EPS molecules. Also, further concentration of the sample substantially improved the detection of a representative number of proteins in gel, as shown by the faint bands of the gels of concentrated samples when compared with pre-concentrated and precipitated samples.

From the precipitation protocols applied to soluble EPS samples, the MALDI-MS results showed that acetone and TCA protocols are complementary, with a set of six or seven proteins, respectively, found to be exclusive to each method in the samples analysed in this study. Protein precipitation explores changes in protein solubility when the solvent suffers perturbations in, for example, the pH and ionic strength. When these perturbations occur, the solvent interacts differently with different proteins, depending on their structure, size, and charge (Evans *et al.*

2009; Englard and Seifter 1990). Therefore, the addition of a ketone or an acid solubilises two distinct sets of proteins and, as such, provides two distinct SDS-PAGE profiles. The results obtained suggest that, for maximal coverage of the extracellular proteins of soluble EPS, these two procedures should be applied in parallel. Perchloric acid-precipitated samples, although resulting in a high number of identified proteins, in general, had higher background and less resolved band patterns than TCA. ProteoPrep® resulted in bigger pellets but not in a higher protein yield, as revealed by the SDS-PAGE gel pattern. In fact, fewer and more faded bands were observed with this method, which reflects a loss of protein content during precipitation. Acetone can also be used to precipitate polysaccharides (Freitas *et al.* 2009), which are the other major components of EPS. Thus, it is expected that the acetone precipitation method co-precipitated polysaccharides that were present in pre-concentrated samples (Table 3.1). However, the results of the SDS-PAGE profiles and the MS results obtained with acetone-precipitated samples resulted in well resolved gels and a high number of identifiable proteins, suggesting that polysaccharides do not interfere with the proteome analysis. For bound EPS samples, precipitation with TCA 13% was considered as the most suited method to achieve an SDS-PAGE pattern of well-resolved bands, i.e. corresponding to identifiable proteins. Nevertheless, the extraction of bound EPS seemed to carry more contaminants than soluble EPS, resulting in stronger smear, although not impairing protein identification. Interestingly, the number of proteins precipitable with acetone was much lower in bound EPS samples than in soluble EPS samples, suggesting that, in the former, proteins were less susceptible to the competition of their polar groups by an organic solvent but responded rapidly to the changes in the pH by the acidic precipitation.

The proteins identified in both EPS fractions (soluble and bound) were similar in molecular function and cellular origin. The vast majority of the proteins identified in this study were described as originally located on the cytosol/cytoplasm subcellular compartment. Three hypotheses can explain these results: these proteins could be 1) natural cell decay products; 2) proteins associated to lysis resulting from the extraction process; 3) proteins that have a function in the EPS matrix, which is currently unknown. Although the reason for this could not be withdrawn from the results obtained, the fact that bound EPS and soluble EPS shared the most abundant proteins identified, indicates that the presence of these three main classes of proteins (structural, elongation factors and transporters) in both fractions are probably not an artefact introduced by the extraction method used, which was different for both EPS fractions.

The outcome of the present study was the elaboration of a workflow that leads to successful protein identification from both soluble and bound EPS samples (Figure 3.4). The sequential concentration and cleaning steps with PP adsorbent gel and acetone/TCA precipitation, allied to a protein separation in acrylamide gradient gels, lead to the visualisation of a complex protein band pattern. Also, the high number of bands with successful identification and the high number of different proteins identified *per* sample and *per* protocol supported the applicability of this approach to environmental, mixed culture EPS proteins. This step-by-step strategy overcame some previously published difficulties that hindered protein analysis in these types of matrices.

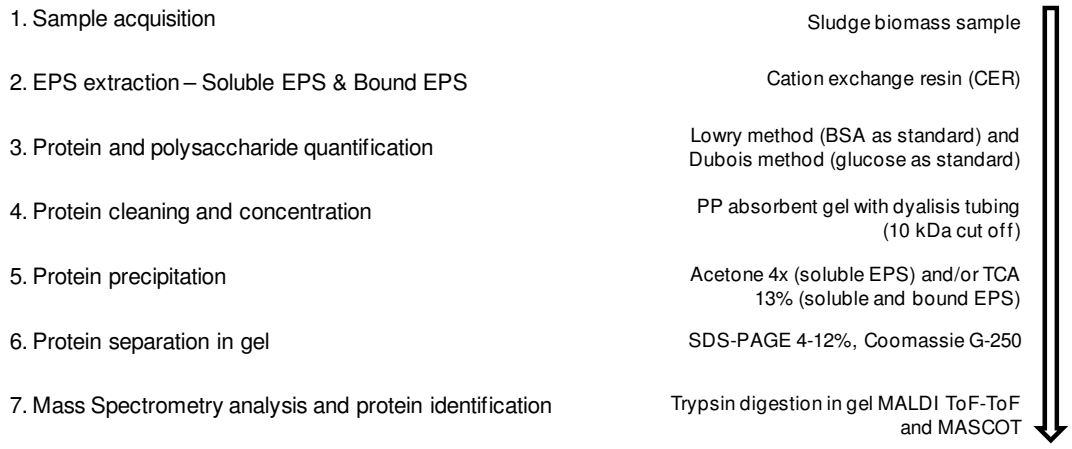

Figure 3.4: Workflow of the proposed protocol for EPS protein analysis. EPS needs to be extracted from activated sludge and separated from cells. Proteins are concentrated and precipitated before application in SDS-PAGE gradient gel. Proteins are then digested in gel and identification is obtained by MALDI ToF-ToF MS and database searching.

The results presented in this paper represent a step forward in the proteome characterization of EPS from wastewater treatment. The proposed method was able to retrieve a higher number of bound EPS proteins than previously proposed methods (Park *et al.* 2008; Higgins and Novak 1997; Denecke 2006), and, for the first time, the identification of soluble EPS proteins was also reported.

In the future, this strategy can be applied to diverse environmental studies where low protein concentration and contaminant compounds, such as humic acids, hinder a comprehensive protein identification.

Acknowledgements

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CHAPTER 4

Microbial population and extracellular polymeric substances diversity in membrane bioreactor under different sludge retention times

Silva AF, Saunders A, Antunes S, Silva C, Vieira A, Barreto-Crespo MT, Carvalho G

Silva AF was involved in all the experimental work presented in this chapter, except for the operation of the lab-scale membrane bioreactor, biomass sampling, data collection (performed by Antunes S and Silva C), protein preparation to be analyzed by MALDI-TOF/TOF and MASCOT search (performed by Vieira A), and metagenomic analysis by next generation sequencing (performed by Saunders A, Department of Chemistry and Biotechnology, Aalborg University, Denmark). The principal component analysis was performed with the support of Cláudia Galinha, FCT-UNL.

CONTENTS

Abstract

Membrane bioreactors (MBR) offer specific conditions to the activated sludge as a consequence of the presence of a physical separation by means of membrane filtration. In this study the microbial community developed in MBRs was characterized as well as the EPS produced by those communities. The microbial and EPS profiles, together with other process parameters, were monitored throughout several months of operation where the reactor was subjected to changes of the sludge retention time (SRT), in order to investigate the impact of this parameter on the diversity and ecophysiology of the biomass. The metagenome of the bacterial community of the mixed liquor and from the cake layer was assessed by Illumina sequencing. The protein profile of the EPS of the suspended flocs was investigated by mass spectrometry (MS). The overall bacterial community structure of the mixed liquor was moderately influenced by changing the SRT from 60 to 20 days. In the cake layer, imposing a shorter SRT lead to a shift in the population, favouring the proliferation and, at ultimately, the predominance of the *Actinobacteria* class. This selection for the *Actinobacteria* class had no parallel in the mixed liquor community, and under the SRT of 20d the cake layer and the suspended flocs populations were significantly distinct. The protein profile of the soluble and bound EPS of the suspended biomass flocs was composed by a high diversity of bacterial protein species, associated to different biological and molecular functions. The majority of the identified proteins are involved in transport and in stress response. The stressful conditions, to which the biomass is apparently exposed, seem to affect less the community structure than protein expression. In the cake layer the stressful environment also impacted in the diversity of the community, with some bacterial groups showing a better adaption capacity to such conditions.

4.1. Introduction

Membrane bioreactors (MBRs) in wastewater treatment combine the biological treatment by activated sludge with a membrane filtration process. This technology presents several advantages over conventional activated sludge (CAS) wastewater treatment processes, together resulting in a high quality effluent, with no need for further disinfection, and a reduction of the footprint of the wastewater treatment plants (WWTP) (Judd 2006).

The efficiency of a biological wastewater treatment process depends on the composition and activity of the microbial community present in the bioreactor. MBRs offer new selective pressures over the activated sludge, among them the total retention of solids, with increased biomass concentration, and the possibility of operating MBRs at long sludge retention times (SRT). These are two operational parameters with great impact on the biomass characteristics, like the diversity of the microorganisms and of the extracellular polymeric substances (EPS) excreted (Judd 2006).

The microorganisms present in MBRs thrive in an aggregative form of life, whether as suspended flocs or as biofilm attached to the membrane. The EPS form the matrix responsible for such aggregation. Besides facilitating cell aggregation, EPS act as a protective environment from external aggressions (dewatering, toxic substances, high temperature and pH) and provide means for easy access to nutrients, for genetic information exchange and for extracellular enzymatic reactions (Flemming and Wingender 2010; Sheng *et al.* 2010; Wingender *et al.* 1999). In some situations EPS can also be cumbersome to engineered systems: an excessive amount of EPS can reduce the mass transfer

processes occurring in wastewater treatment plants (Sheng *et al.* 2010). Moreover, the permeability of MBRs is severely affected by the accumulation of EPS at the membrane surface and pores, which results in increased operation and capital costs (Drews 2010).

EPS is composed of several macromolecules including proteins, polysaccharides, humic acids, extracellular DNA and other polymeric compounds such as glycoproteins and glycolipids (Flemming *et al.* 2007). These molecules are produced and excreted by microorganisms, or released as a result of cell lysis, and also include other organic molecules present in the wastewater that adsorb to the flocs (Wingender *et al.* 1999). EPS can be divided into two fractions: soluble and bound EPS, in which soluble EPS is the set of biopolymers and colloids dissolved in solution, whereas bound EPS is the set of biopolymers that are closely bound to the microbial cells (Sheng *et al.* 2010; Wingender *et al.* 1999).

Until recently the molecular tools available permitted only the assessment of the most represented microbial communities of the population. Most of these studies in MBRs focused on the microbial consortia involved in biological nutrient removal such as the nitrifiers and the polyphosphate accumulating microorganisms and direct competitors groups (Silva *et al.* 2012a; Miura *et al.* 2007). With the implementation of advanced techniques such as next generation sequencing (e.g. 454 Pyrosequencing, Illumina), broader descriptions of the microbial community in MBRs have been obtained in the last two years (Saunders *et al*. 2013; Ma *et al.* 2013; Lim *et al.* 2012; Kim *et al.* 2013). Differences between MBRs and CAS have been pointed out, as well as between different zones inside the MBRs (in the mixed liquor or at the membrane). Nevertheless, despite the much deeper population characterization achieved with these techniques, many

of the sequences are still classified as uncultured or unidentified by the available databases.

The knowhow about EPS composition in MBRs is also very scarce, as a consequence of the knowledge gap concerning the microbial populations. Recent progress was made with metaproteomic approaches and several proteins species were described (Silva *et al.* 2012b; Miyoshi *et al.* 2012; Huang *et al.* 2012). This kind of reports is starting to uncover the complexity of the microbial population and EPS diversity but much is yet to unravel. Moreover, little is known about the influence of operational parameters and conditions on community structure and on the extracellular protein profile.

The aim of this study was to elucidate the effect of imposing a high sludge retention time, as often used in MBRs, on the bacterial population and corresponding production of EPS, as major fouling agent.

The biomass was subjected to two different SRT and sampled along time to assess the impact of the change in condition on the diversity and ecophysiology of the populations. Molecular information obtained using Illumina technology, and proteomics to identify the EPS proteins, were combined with process data to investigate the correlation between MBRs operational conditions, microbiology and performance. This information is expected to give a step further in the understanding the microbial selection and activity in MBRs in different conditions, which ultimately may contribute to advance operation/design for improved performance.

4.2. Material and Methods

4.2.1. Membrane bioreactor

The activated sludge samples were collected from a MBR comprising a 13 L anoxic tank and a 17 L aerobic tank, with a submerged module of polysulphone HF membranes (Polymem, Tolouse, France), with an area of 0.1 m^2 and a pore diameter of 0.1 µm. The reactor was fed with municipal wastewater collected after primary settling. The feed was supplemented to maintain a food-to-microorganism (F/M) ratio constant, using sodium acetate, ammonium chloride, hydrogen potassium phosphate and dihydrogen potassium phosphate in a proportion 100 mgCOD: 5 mgN: 1 mgP. The aeration flow rate was set at 40 L min^{-1} to promote membrane scouring. The operational conditions are listed in Table 4.1 with average values throughout the experimental period. The transmembrane pressure (TMP) was continuously acquired on-line (pressure transmitter PTX1400, Druck). Membrane was chemically cleaned when the TMP was above 700 mbar. To determine the influence of the SRT on the microbial population, the MBR was first operated at a SRT of 60 days followed by a SRT of 20 days.

Table 4.1: Operating parameters (average and standard deviation) for the MBR during the two experimental periods

	60 d SRT	**20 d SRT**
HRT (h)	35 ± 13	44 ± 21
TMP (mbar)	297 ± 161	523 ± 207
F/M (h^{-1})	0.01 ± 0.00	0.01 ± 0.01
MLSS (gL^{-1})	5 ± 1	7 ± 1
VSS (gL^{-1})	4 ± 1	6 ± 1
pH	8.0 ± 0.5	8.0 ± 0.5
Temp (°C)	20 ± 2	20 ± 2

HRT: hydraulic retention time; TMP: transmembrane pressure; F/M: food to microorganisms ratio; MLSS: mixed liquor suspended solids; VSS: volatile suspended solids

Activated sludge samples were collected from the mixed liquor (named ML) and cake layer (named CL) for DNA and EPS extraction throughout the study.

4.2.2. Metagenomic studies

The DNA was extracted from 1 mL of sludge with a MoBio Ultraclean™ soil DNA kit (Cambio, Cambridge, UK) according to the manufacturer's instructions with a minor adaptation in the beginning of the protocol: samples were centrifuged at 10 000 rpm for 5 min and the supernatant was rejected. The Bead Solution included in the kit was added to the sample pellets and vortexed to mix thoroughly. The remaining steps fully respected the instructions.

Community composition was investigated using high throughput sequencing. PCR amplifications were made with Platinum® High Fidelity Taq Polymerase (Invitrogen, USA) in two-steps: 1) 20 PCR cycles with 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) and 1-10 ng of template DNA, 2) 7 PCR cycles using the same primers plus a 12 nt barcode and Illumina adaptors (Caporaso et al. 2012) using 2 µL of the first product as template. DNA concentration was measured with QuantIT (Molecular Probes, Germany) and barcoded amplicons were pooled in equimolar amounts and sequenced on an HiSeq 2000 (Illumina, USA). QIIME (qiime.org) was used for OTU clustering and taxonomic assignment (RDP Classifier using the Midas database (www.midasfieldguide.org) and additional analyses and visualizations were made in R with the phyloseq (McMurdie and Holmes 2013) and the vegan package (http://CRAN.R-project.org/package=vegan).

4.2.3. Identification of EPS proteins

Soluble EPS was obtained by collecting the supernatant after centrifuging 400 mL of activated sludge (12000 g, 15 min, 4 ºC). The bound EPS was extracted through incubation with a cation exchange resin (CER) (Dowex® MARATHON®, 20-50 mesh, Sigma Aldrich, St Louis, USA) according to Frolund *et al.* (1996) with minor modifications. Total protein content was determined by the Lowry method (Frolund *et al.* 1996), using bovine serum albumin (BSA, Merck KGaA, Germany) as standard. Total polysaccharide content was determined by the Dubois protocol (Dubois *et al.* 1956), and calibration was performed with glucose (Sigma chemical Co, St Louis, USA). Measurements were performed in an Ultrospec 2100 pro

spectrophotometer (Biochrom Ltd, Cambridge, England) and all samples were measured in duplicate.

Identification of EPS proteins were conducted as described by Silva *et al.* (2012). In summary, for soluble EPS proteins, samples were concentrated overnight and precipitated in parallel with acetone 4× and trichloroacetic acid (TCA) 13 %. For bound EPS proteins, the same protocol was applied but just with the acidic method. The protein pellets were resuspended with NuPAGE® LDS sample buffer (4×) (Invitrogen, Carlsbad, CA, USA). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the NuPage® electrophoresis system instructions (Invitrogen, Carlsbad, CA, USA). The gel bands of interest were excised and in-gel-digested with trypsin as described (Santos *et al.* 2009). The procedure to identify the proteins by Maldi ToF/ToF is described elsewhere (Silva *et al.* 2012b). When one fragmented peptide was not obtained, protein identification was accepted if the protein score was above the MASCOT score (83 for NCBInr and 70 for SwissProt), with 95% confidence for both databases, as long as the database matched more than two peptides.

Principle component analysis (PCA) was used to correlate the identified proteins with the operational parameters and EPS fractions. PCA was implemented in Matlab using the PARAFAC routine (Andersson and Bro 2000 ; Bro 1997).

4.3. Results and Discussion

4.3.1. MBR operation and sampling

The MBR was operated at two different SRTs: a first period of 60 days (278 days of operation) followed by the substitution of the membrane module and the beginning of a new period (178 days of operation) were the SRT was 20 days. For the period with SRT of 60 days, sampling started after a period of three SRTs to allow the reactor to achieve a pseudo steady-state. For the period with SRT of 20 days three samples (ML6 and 7, and CL8) were taken during the adaptation period and three (ML9 and 11, and CL10) after three SRTs at pseudo steady-state. The TMP was in average higher during the 20d-SRT period, which indicates an overall higher level of fouling of the membrane. These results agree with previous works. Van den Broeck *et al*. (2012) described lower fouling rates at higher SRTs (30 and 50 days). Su *et al*. (2011) also observed that an increase from an SRT of 10 days to 60 days was reflected in a decrease in TMP.

4.3.2. Phylogenetic analysis of suspended and cake layer biomass

Biomass samples were collected in different dates simultaneously from the suspended mixed liquor and the biofilm attached to the membrane (cake layer). DNA was extracted to perform a comprehensive microbial population analysis by high throughput sequencing with Illumina technology. A total of 102,300 sequence reads with valid bar codes were generated from the eleven samples, grouped in 1372 OTUs, most of them classified at the genus level.

Figure 4.1 summarizes the microbial composition at different taxonomic levels: the major phyla of the 10 most abundant OTUS and the major genera of the 15 most abundant OTUS in the mixed liquor (ML) and the cake layer (CL) biomass.

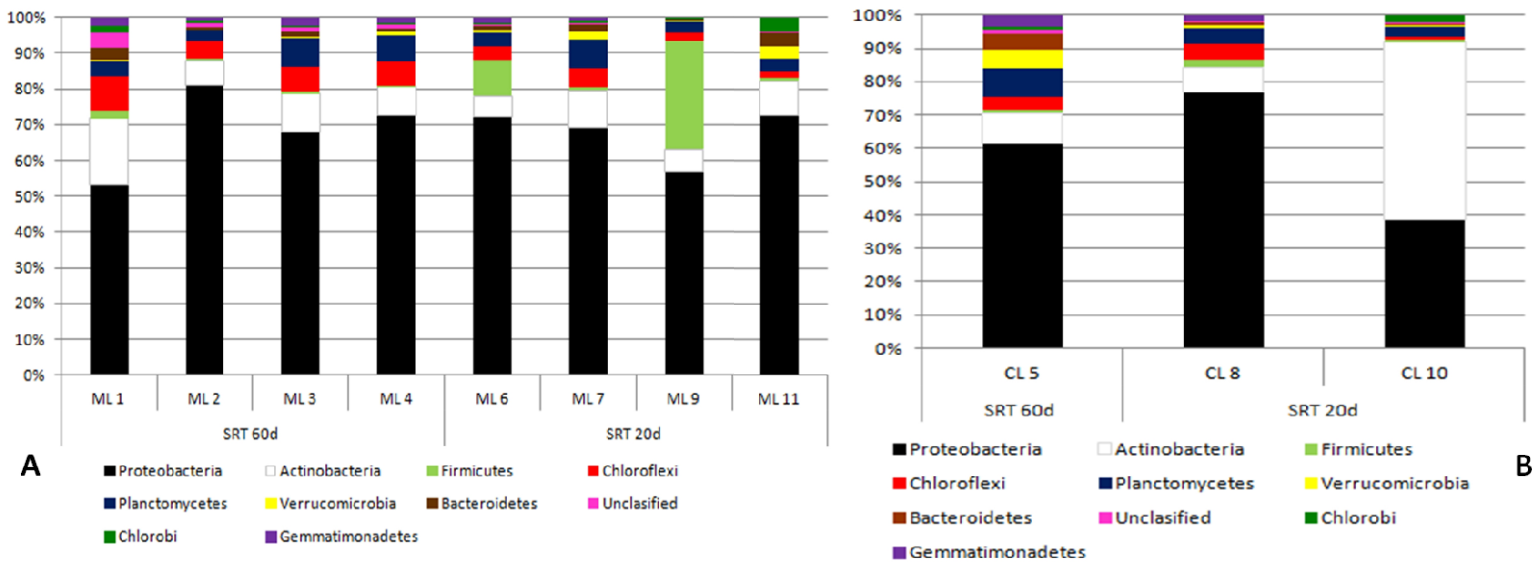
The microbial population in the mixed liquor was very dynamic, where different communities were dominant at different operation times. Throughout the two operation periods, *Proteobacteria* was the major phylum in the mixed liquor, with an average abundance of 67%. Within *Proteobacteria*, *Alphaproteobacteria* was always the preponderant class followed by the *Gammaproteobacteria*, except for samples ML2 where *Gammaprotebacteria* alone accounted for 57% of the total microbial population. This preponderance was accomplished by the bloom of the filamentous bacteria belonging to the *Thiothrix* genus (55%) in that date. *Thiothrix* was always present in the mixed liquor during the period operated at higher SRT, with abundances between 4 and 18 %, except for the mentioned bloom. When the SRT was reduced to 20 days, the percentage of these organisms decreased during the adaptation period until none (0%) is detected after the new SRT was established. Apart from the *Thiothrix*, a high diversity of other genera of *Gammaproteobacteria* and *Alphaproteobacteria* were observed. *Actinobacteria* were present throughout the two periods in similar percentages, oscillating between 5% and 18%, and a high diversity of genera was identified within the *Actinobacteria* class. Unidentified genus from the *Actinomycetales* order was the predominant actinobacterium. Members of the *Chloroflexi* and *Planctomycetes* phyla were also present in both operation periods in similar proportions. The *Firmicutes* phylum had low abundance during the 60d-SRT period, comprising a maximum of 2% of the population, but when reducing the SRT to 20 days, it showed a very unsteady presence both during the adaption

period as in the established 20d-SRT period, due to the proliferation of microorganisms from the *Lactobacillus* genus (ML6) and the *Carnobacterium* genus (ML9). Unclassified bacteria were more preponderant in the period of 60d-SRT. *Bacteroidetes* and *Chlorobi* had a constant presence, with low but variable abundances, throughout the two periods. Bacteria from the *Gemmatimonadetes* and *Verrucomicrobia* were present in low proportions but with a higher proliferation in the longer (*Gemmatimonadetes*) or shorter (*Verrucomicrobia*) SRT periods.

The change in SRT imposed in this study did not modify the overall microbial distribution of the suspended flocs at the phylum level and the 15 principal OTUs varied mostly in their relative abundances along each operation period. At this taxonomic level our results are in agreement with other MBRs studies comparing microbial communities at different SRT periods, in which the bacterial phyla present in the mixed liquor at an SRT of 10 days and 60 days were very similar (Su *et al.* 2011). Our results also are in agreement with Lim *et al.* (2012), in which the population in the mixed liquor, subjected to a SRT of 30 days and after acclimatization was mainly composed by *Proteobacteria*, classes *Gamma*- and *Alphaproteobacteria*, and *Actinobacteria.*

The bacterial community of the cake layer was also diverse and showed more accentuated differences between the two SRT periods. The top most abundant OTUs detected in the cake layer during the 60d-SRT period had a much broader diversity of phyla than at 20d-SRT. *Proteobacteria* was the predominant phylum for longer SRT (60%) and the adaptation period between both SRTs (76%), but when the SRT of 20 days was established the proportion of *Proteobacteria* dropped (38%). *Alpha-*, *Gamma*- and *Betaproteobacteria* were the three classes with most significant abundance within the *Proteobacteria* phylum.

Alphaproteobacteria was always the most predominant class among the phylum in the three samples. *Betaproteobacteria* (13%) in the longer SRT period outgrew *Gammaproteobacteria* (9%), but in the adaptation period (sample CL8) proportion values dropped (4%) and stabilized throughout the shorter SRT period. During the adaptation period (sample CL8) *Gammaproteobacteria* abundance increased temporarily, but fell back to former values when that SRT was established. *Deltaproteobacteria* gained relevance in the CL5 sample (5%), mainly composed of an unidentified genus of the family *Polyangiaceae*, but disappeared (0%) with the changing of SRT. Several genera from the *Alphaproteobacteria* class were present, and three genera from the *Gammaproteobacteria*, namely *Thiothrix*, *Pseudoxanthomonas* and *Thermomonas*. *Thiothrix* had a significant development during the adaptation period (10%) but disappeared (0%) from the cake layer in the CL10 sample.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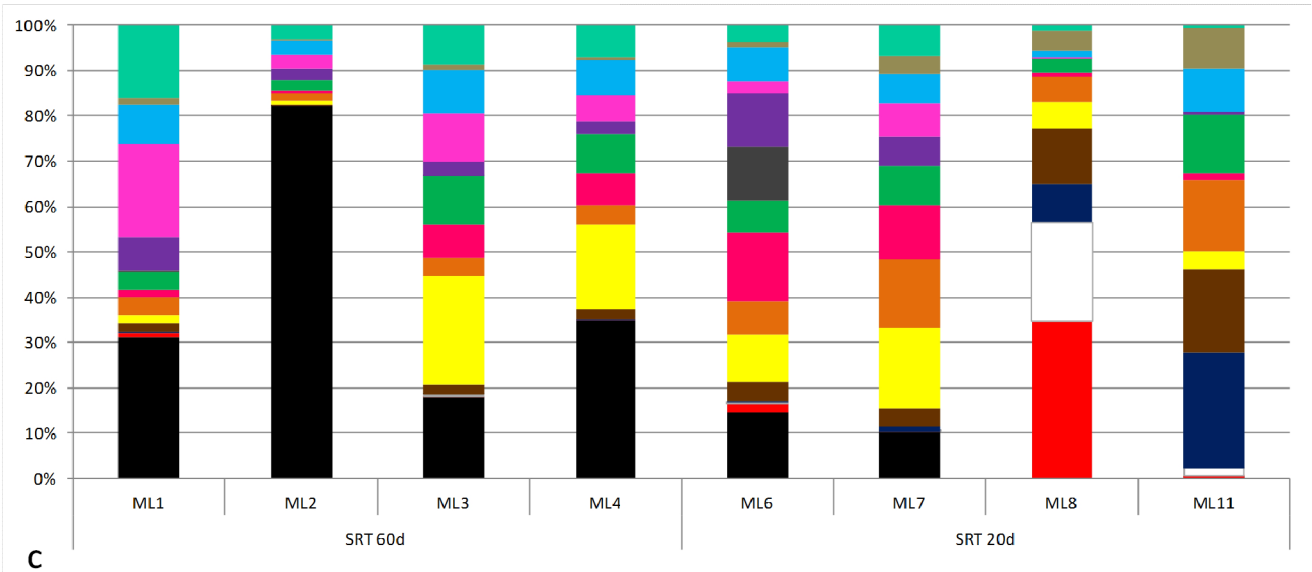
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k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Psychrobacter
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthamonadaceae;g__Pseudoxanthomonas
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k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__Mesorhizobium
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthamonadaceae;g__Thermomonas
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Ellin;f__UnkEllin;g__Unk01
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;Other
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__;g__
k__Bacteria;p__Chloroflexi;c__Thermomicrobia;o__HN1-15;f__;g__

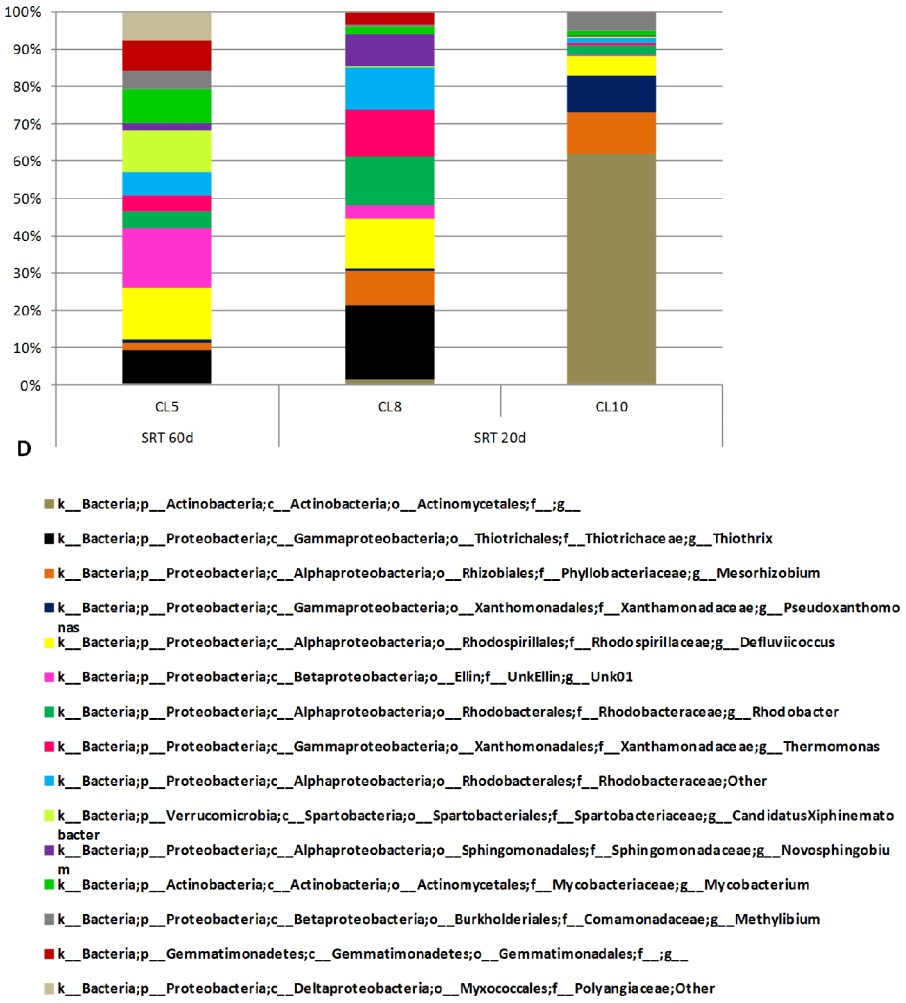


Figure 4.1: Phylogenetic distributions throughout the two operation periods at different sequential sludge retention time (SRT) of the biomass from the mixed liquor (ML) and the cake layer (CL): A) and B) the top 10 most abundant OTUS of the major phyla, and C) and D) the top 15 most abundant OTUs for the major genera. Pseudo steady-state samples: ML1-4, ML9 and ML11; CL5 and 10. Adaptation period samples: ML6 and 7; CL8

The 20 d SRT favored the proliferation of the *Actinobacteria* phylum (53%) in the cake layer, mainly due to the increase in the abundance of an unidentified genus from the *Actinomycetales* order (49%), which dominated the biofilm formed at this stage. The *Firmicutes* phylum was always present in low (1-2%) and constant proportions during the two SRT periods. *Chloroflexi*, *Planctomycetes* and *Bacteroidetes* phyla had a constant presence in the three cakes but where more represented in the longer SRT (CL5: 18%; CL8:10%; CL10: 5%). *Verrucomicrobia*, *Gemmatimonadetes* and bacteria not identified in any phylum were present in low proportions at 60d-SRT but reached values close to 0% at 20d-SRT. Regarding the distribution of the different genera throughout the two operation periods, an enormous diversity was observed in the bacterial population enriched at 60d SRT, covering all but one representative from the most important 15 OTUs identified in this study. Only a new genus, *Pseudoxanthomonas* from the *Gammaproteobacteria* class, appeared between the 15 most important OTUs in the cake layer sample taken when the reactor was at a pseudo steady-state with the SRT of 20 d.

The SRT seemed to impact the structure of the microbial population of the cake layer, in which there are differences seen both at the phylum level as at the genus level, with OTUs appearing only in one of the operation periods. The changing for a shorter SRT seemed to turn the cake layer biomass in a more selective environment where a smaller group of bacterial genera outcompeted the others. This was not observed in the suspended biomass, where a higher number of genera were always observed, distributed in a more even manner, except for *Thiothrix* in sample ML2. Jinhua *et al.* (2006) had previously reported that a higher diversity was observed in a MBR mixed liquor whereas in the cake layer the microbial population was more restricted, and speculated that this

attached population was composed of specialized members for the formation of the biofilm. Lim *et al.* (2012) also analyzed the community structure of a MBR biocake under a SRT of 30 days and found that the bacterial community was more restrained, with a family of microorganisms dominating the overall structure.

Comparing the population between the mixed liquor and the corresponding cake layer at the SRT period of 60d (ML4 and CL5), at the phylum level the populations of both MBR zones were similar: the phyla with higher proportions were similarly present in the two samples and the remaining were equally or more predominant in the cake layer. The same was observed for the genera, in which only *Pseudoxanthomonas* and the unclassified *Betaproteobacteria* Unk01 had a higher proportion in the cake layer sample. The bacterial population during the pseudo steady-state of the operation period of 20 d SRT showed clear differences between the suspended flocs and the cake layer. *Actinobacteria* dominated (53%) the cake layer bacterial population, mainly composed of the unidentified genus from the *Actinomycetales* order (49%), which in the mixed liquor only represented 6% of the population. *Proteobacteria* was abundant in both samples, but were more important in the mixed liquor. Among the 15 most abundant OTUs, the genera from the class *Gammaproteobacteria* in common between the two types of populations were only the *Pseudoxanthomonas* and *Thermomonas*. *Psycrobacter* genus was only present in the mixed liquor. Some genera from the *Alphaproteobacteria* were only seen in the cake layer (*Methylibiu*) or in the mixed liquor (unidentified genus from the *Caulobacteraceae* family). *Firmicutes* in the cake layer only represented 1%, but in the mixed liquor it was the second most representative phylum, with a proportion of 30%. The remaining genera were present in both samples in similar proportions.

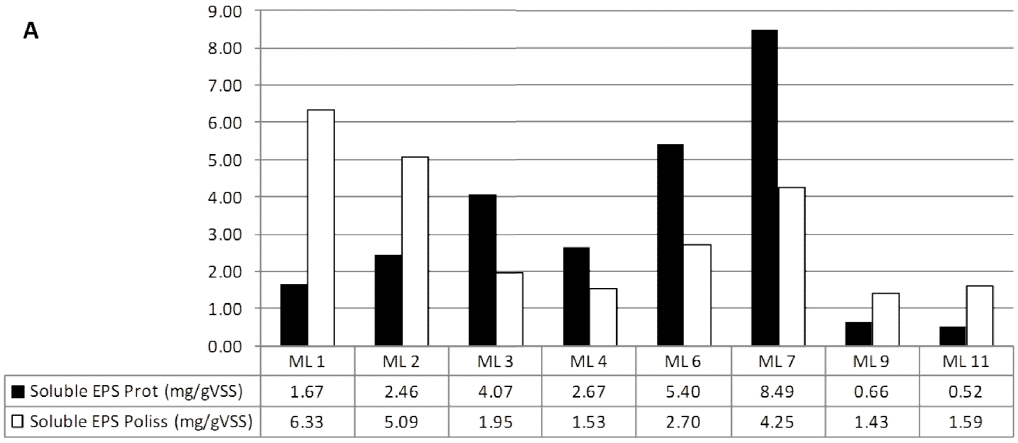
In sum, the bacterial community established in the mixed liquor under the SRT of 20 days was substantially different from the cake layer. These results are in agreement with a study comparing suspended and cake layer population at a SRT of 30 days where it was found that the community structure of the biocake was clearly different from the mixed liquor and that only a few major players in the whole microbial community composed the biocake (Lim *et al.* 2012).

4.3.3. Extracellular proteome of the suspend flocs

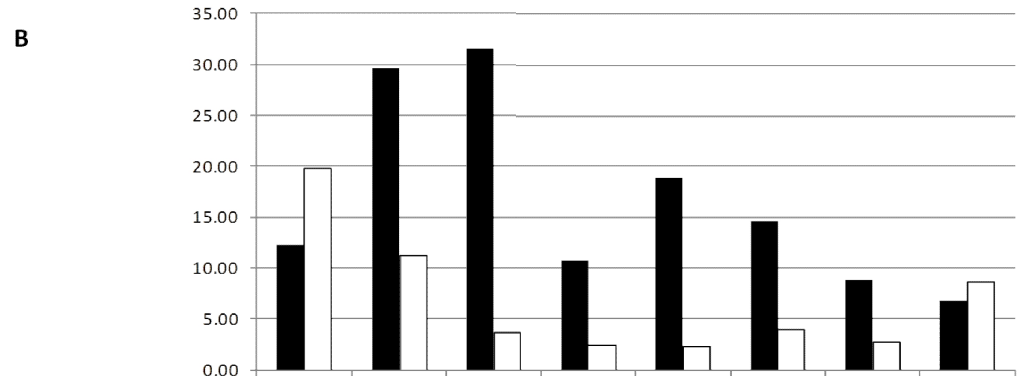
4.3.3.1. Protein and polysaccharide content of soluble and bound EPS

The concentrations of proteins and polysaccharides were measured for each fraction of EPS from the mixed liquor samples (Figure 4.2). The protein to polysaccharide ratio was variable throughout the study. Protein concentration in the soluble EPS increased during the adaptation period of the microbial population to the new SRT of 20 d. The same trend was followed by the polysaccharide concentration, although it was not so pronounced. Bound EPS had a higher concentration of proteins than soluble EPS, normally with a protein to polysaccharide ratio higher than 1. In the case of bound EPS, the protein content was higher during the 60 d SRT period with a sharp decrease close to the end of this period. During the adaptation to the new SRT of 20 d, the protein content also had an increase like for the soluble EPS, although more discrete, to further drop to low values. Analyzing the total protein and polysaccharide concentration from both EPS fractions for each SRT period, in average proteins (60 d SRT: 137 ± 54 mg $gVSS^{-1}$; 20 d SRT: 78 ± 31 mg $gVSS^{-1}$) were more abundant than polysaccharides

(60 d SRT: 50 ± 30 mg $gVSS^{-1}$; 20 d SRT: 38 ± 14 mg $gVSS^{-1}$). The EPS concentration of both molecules was higher in the first SRT period.



	ML 1	ML 2	ML 3	ML 4	ML 6	ML 7	ML 9	ML 11
■ Soluble EPS Prot (mg/gVSS)	1.67	2.46	4.07	2.67	5.40	8.49	0.66	0.52
□ Soluble EPS Poliss (mg/gVSS)	6.33	5.09	1.95	1.53	2.70	4.25	1.43	1.59



	ML 1	ML 2	ML 3	ML 4	ML 6	ML 7	ML 9	ML 11
■ Bound EPS Prot (mg/gVSS)	12.31	29.58	31.60	10.80	18.95	14.65	8.85	6.83
□ Bound EPS Poliss (mg/gVSS)	19.84	11.20	3.68	2.41	2.37	3.96	2.70	8.57

Figure 4.2: Protein and polysaccharide quantification results from soluble (A) and bound (B) EPS from mixed liquor samples taken throughout the two periods of MBR operation, with an SRT of 60 days (samples ML1-4) or 20 days (ML6-11)

4.3.3.2. Protein identification from soluble and bound EPS

With the metaproteomic approach followed in this study, a total of 148 bacterial proteins were identified from the EPS of suspended flocs. A higher number of gel bands (225) were excised and prepared for mass spectrometry analysis. This imbalance could result from several factors:

- Some protein hits were from eukaryotes with several possible origins, as for example from wastewater input, contamination or minor representatives of the activated sludge;

- Some gel bands were faint. Protein concentration were likely near (when a small number of identifications were obtained) or below (when no identification was obtained) the detection limit of the equipment;

- Gel bands were strong but no identification was retrieved. The majority of the missing identifications were due to the lack of database matching. Most of EPS proteins are expressed by uncultured or unidentified bacteria, thus reducing the probability for coverage by the public protein databases;

- Also, some proteins may have post-translational modifications that impair the ionization of the peptides in the MALDI ToF/ToF, and, when it was possible to retrieve identification from the database search it was only based on the number of matching peptides.

Among the protein species identified, a large diversity was observed (Figure 4.3). The number of proteins and the diversity were lower in the 20d-SRT period.

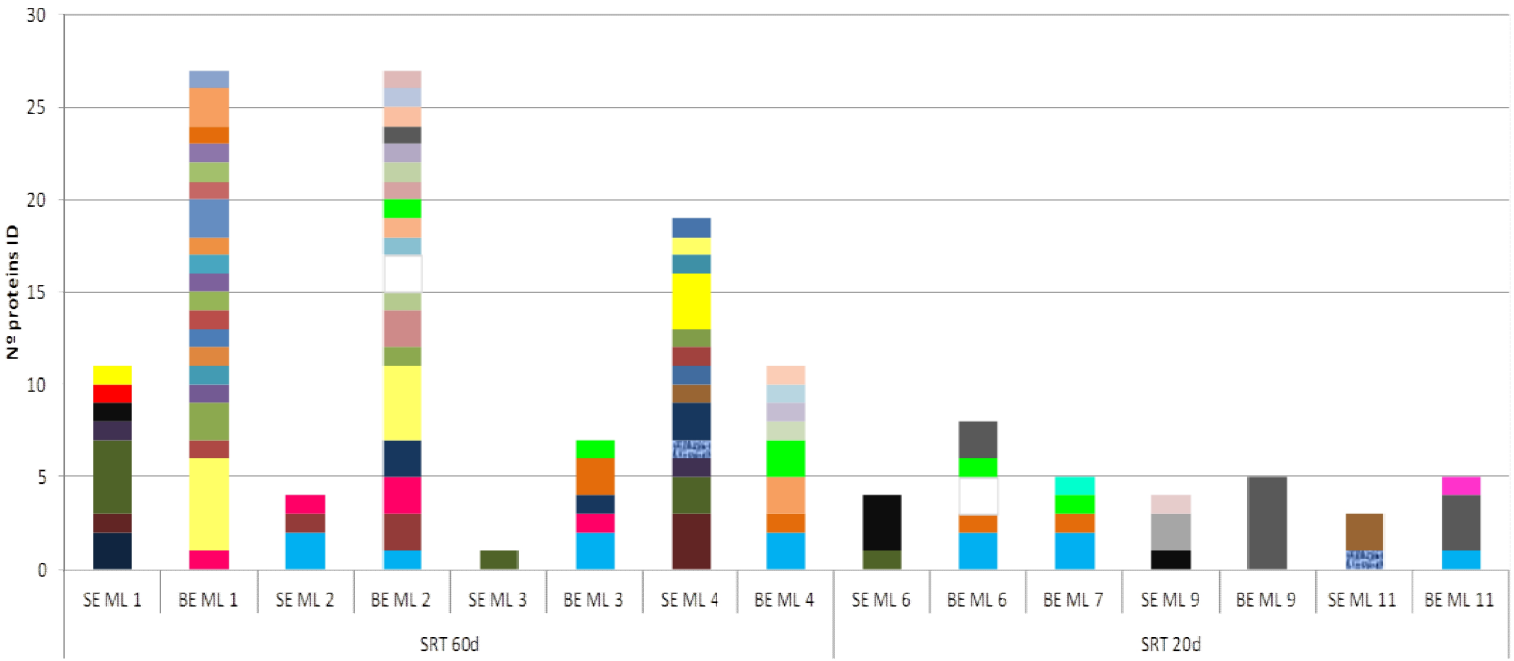

Nº proteins ID
30
25
20
15
10
5
0
SE ML 1
BE ML 1
SE ML 2
BE ML 2
SE ML 3
BE ML 3
SE ML 4
BE ML 4
SE ML 6
BE ML 6
BE ML 7
SE ML 9
BE ML 9
SE ML 11
BE ML 11
SRT 60d
SRT 20d

Figure 4.3: Protein species identified from soluble (SE) and bound (BE) EPS extracted from samples taken from the mixed liquor (ML) throughout the two MBR operation periods, at SRT of 60 or 20 days

Analyzing the proteins grouped by their described molecular functions (Figure 4.4) and biological processes they are associated to (Figure 4.5), it was found that a significant part was involved in stress response, transport and biosynthesis. Lyases are described as extracellular enzymes involved in polymer degradation (Wingender *et al.* 1999). The degradation of extracellular polymeric substances (EPS) can occur to provide nutrients for the sessile bacteria, but also as a strategy in stress situations to free the bacteria from such environment (Flemming and Wingender 2010). Other proteins with a defined task in the cell, as the ABC transport type system, also have a role in stress tolerance (Seaton *et al.* 2011). Oxireductases and phosphatases, found in our study, are also commonly found as extracellular enzymes in biofilms (Wingender *et al.* 1999; Flemming and Wingender 2010). Proteins in EPS may also have a structural function, being involved in several features of the EPS, such as adhesion, protection and aggregation. Lipases in EPS can behave as structural components being involved in the formation and stabilization of the EPS matrix, performing the link between the cell surface and extracellular EPS (Wingender *et al.* 1999).

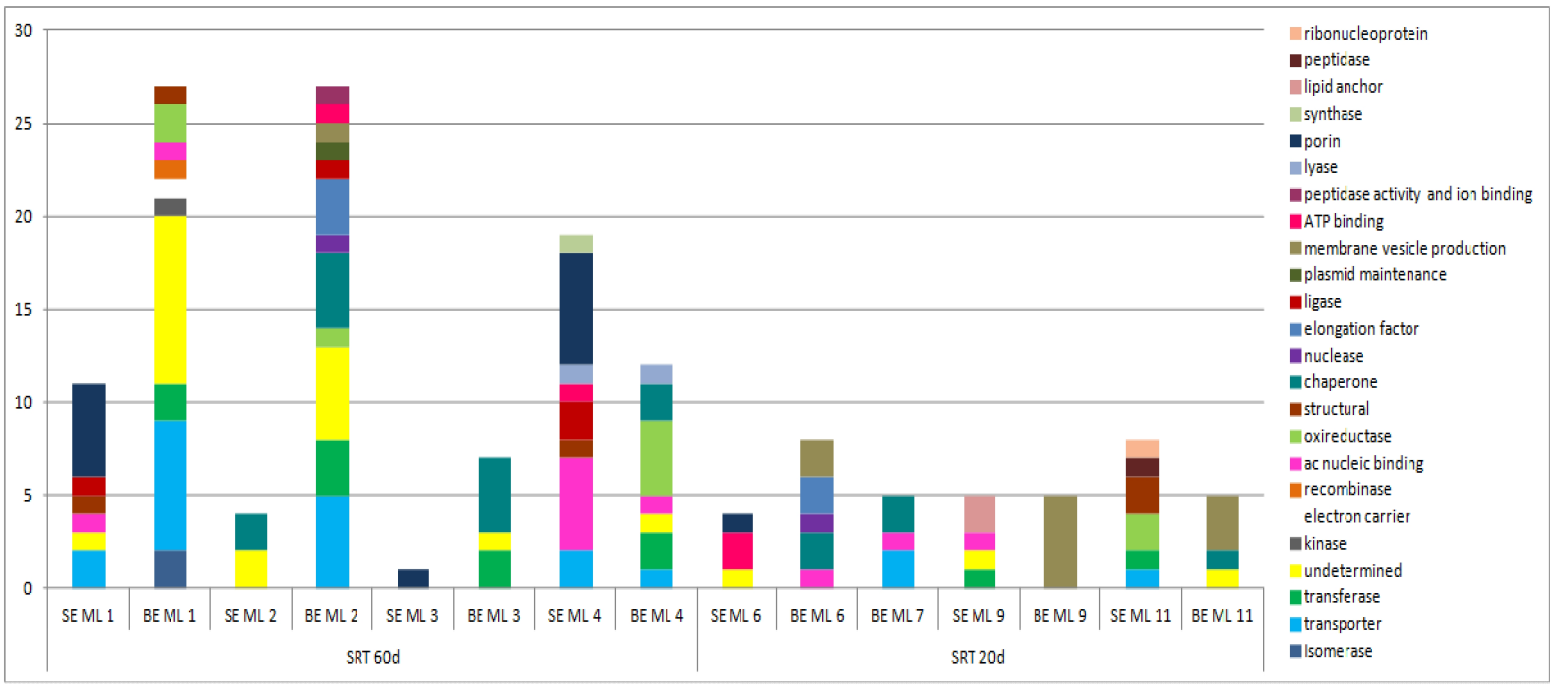


Figure 4.4: Protein identified from the soluble EPS (SE) and bound (BE) EPS extracted from samples from the mixed liquor (ML) of the MBR. Proteins species were grouped by molecular function, according to Protein Knowledgebase UniProtKB/Swiss-Prot database

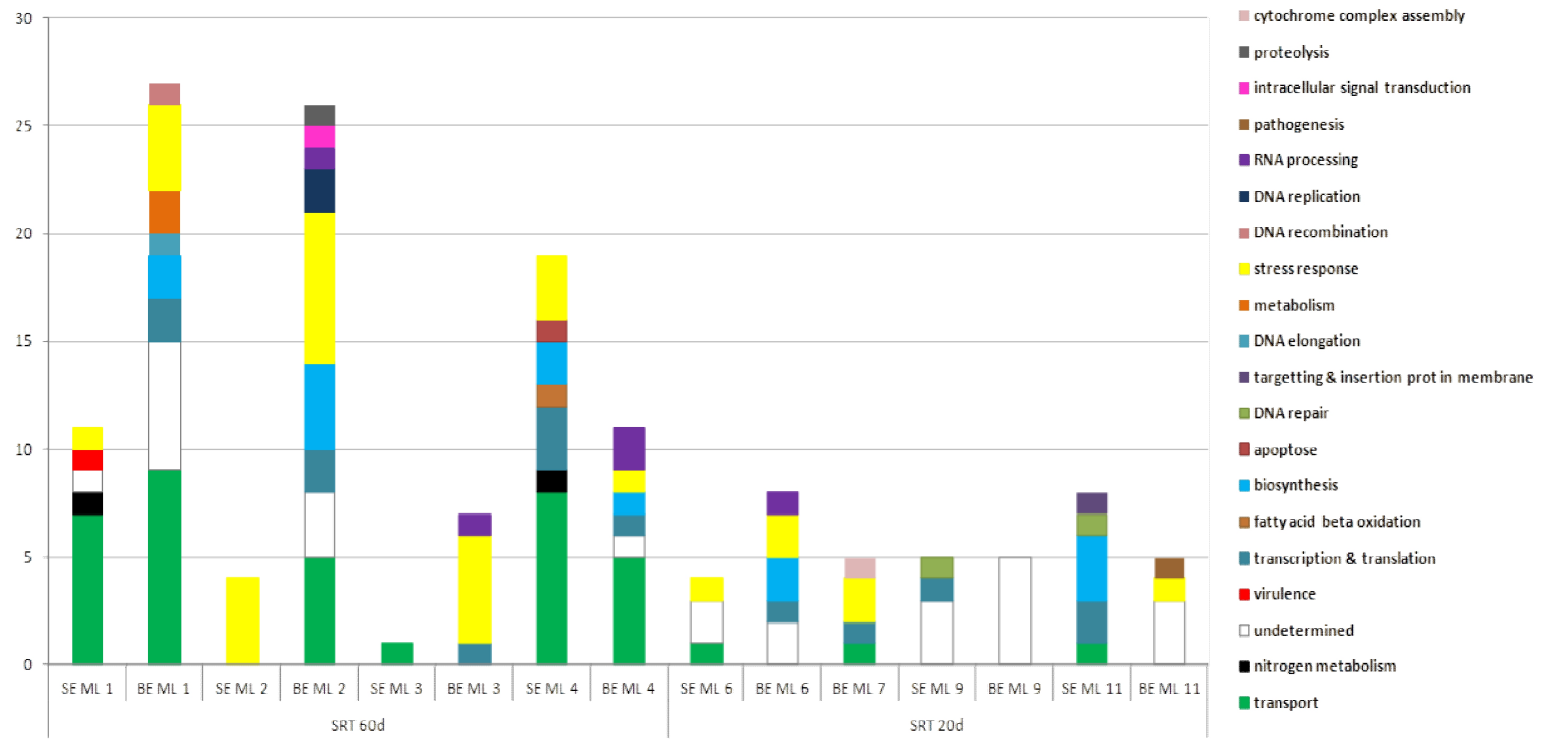


Figure 4.5: Protein identified from the soluble EPS (SE) and bound (BE) EPS extracted from samples of the mixed liquor (ML) of the MBR. Proteins species were grouped by biological processes they are associated to, according to Protein Knowledgebase UniProtKB/Swiss-Prot database

PCA was used to investigate the existence of correlations between protein profiles, the operational conditions and EPS fractions. The soluble EPS protein profiles clearly differed from those from bound EPS (Figure 4.6A). Only one soluble EPS sample (SE ML2) clustered with the bound EPS samples. This possibly reflects some bias input by the extraction procedure or by conditions inside the bioreactor at that time date, making the protein profile similar between the two EPS fractions of that sample.

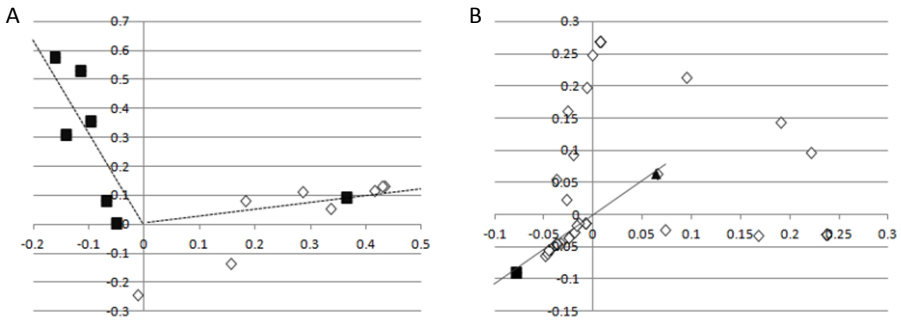


Figure 4.6: PCA analysis of the EPS protein profile regarding the A) soluble (■) and bound (◇) EPS fraction and the B) relation between the protein profiles (◇) with EPS fraction (■) and SRT (▲)

A group of 14 proteins correlated well with the corresponding EPS fraction and also with the SRT where they were found, with a captured variance of 51.6% (Figure 4.6B). These 14 proteins (Table 4.2) were only identified in soluble EPS samples, never detected on the bound EPS counterpart. They were also mainly detected in samples taken during the 60 d SRT period (SE ML1, SE ML2, SE ML4), although SE ML11 also contained three of these proteins.

Table 4.2: Protein species identified from soluble EPS samples taken throughout the two operational periods

Protein species	Sample
Peptidoglycan-associated lipoprotein	SE ML1
Cold shock protein	SE ML1/2
Outer membrane porin F (OprF)	SE ML1
Glutamine synthetase	SE ML4/11
Membrane protein	SE ML1/6/9
Outer membrane protein (OmpA)	SE ML 1
ATP synthase	SE ML4/11
50S ribosomal protein (large subunit)	SE ML4/11
Fatty acid oxidation complex	SE ML4
Succinyl-CoA ligase [ADP-forming]	SE ML4
Outer membrane protein (Omp38)	SE ML4
Putative OprD protein	SE ML1/4
Malate-CoA ligase	SE ML4
AapJ	SE ML4

SE: soluble EPS; ML: mixed liquor

In light of the PCA results, we further compared in detail the protein profiles between soluble EPS and bound EPS from the same date. Regarding the cellular location of the identified proteins, bound EPS has a major content of proteins described in the protein database as intracellular. Regardless if they are extracellular enzymes or if they are lysis products, their presence in bound EPS is to be expected because this is the fraction more closely bound to the cells, thus trapping in first place the enzymes, lyses and leakage cell products. Even so, the products from natural decay are also conventional classified as components of the EPS, and can be regarded by the microorganisms composing the aggregates as nutrient sources, for example (Wingender *et al*. 1999). The proteins from the membrane, transmembrane or outer proteins were extracted in both EPS fractions in equivalent proportions, which probably depended on how strongly they were attached to the cell membrane.

Concerning the molecular function and biological processes that the identified EPS proteins are involved in, there are molecular functions only present in the soluble EPS fraction. Porins are passive transporters and were only detected in the soluble EPS, but active membrane transporters were found in both fractions. One can postulate that porins are present in the soluble EPS due to cell debris after lysis or due to intentional secretion. The secretion of porins was described for example for *Borrelia burgdorferi* and referred as an important component of the host-pathogen interaction (Cluss *et al.* 2004). Among other macromolecules, the EPS is composed also of extracellular DNA (eDNA), actively excreted by the cells. Therefore, the presence of DNA binding proteins in the soluble EPS was expected (CApB protein, cold shock protein, DNA-directed RNA polymerase subunit alpha and ARG family of transcriptional regulator). Their absence in the bound EPS may indicate that eDNA has a structural function not bound to the cells, but to the remaining molecules of the matrix. The virulence protein detected only in the soluble EPS can indicate a mechanism of defence or the presence of infectious bacteria brought with the wastewater. Enzymes involved in the biosynthesis pathways and synthesis of nucleic acids and proteins were exclusively found in bound EPS. Proteins involved in response to stress, transport and metabolism were observed in both EPS fractions but with more relevance in the bound EPS fraction. These types of protein classes are important in biofilm maintenance through nutrition and protection (Flemming and Wingender 2010), and it seems that they are maintained very close to the cell. The proteins exclusive to soluble EPS seem more related to the structure of the matrix and to the defense of the biofilm.

To the best of our knowledge it is the first time that a comprehensive analysis of the microbial population and the extracellular proteome present in

MBRs is presented. The microbiota of MBRs is very diverse, with representatives of many different phyla, and dynamic, varying their abundances throughout operation and in different locations inside the reactor, probably adapting themselves to the different conditions that the microorganisms had to face in such environment. The metaproteome from the suspended flocs from both operational periods was highly diverse, with proteins enrolled in different functions. Although most of these functions are described in the database hits as occurring inside the cell, the presence of these proteins in the extracellular medium does not necessarily imply that all came from cell lysis. Furthermore, the biomass was subjected to different operational conditions, typical of MBRs operation, to study the impact on the microbial ecophysiology. Analysing the impact of SRT on the combined results of the microbial profile and the extracellular proteome, the SRT seemed to have a contribution on the different microbial community structure of the cake layer and to affect the protein profile of the soluble EPS.

The bacterial populations seem to be facing a stressful environment in the mixed liquor, not reflected in the concentration of the EPS components but in the protein species expressed. In the cake layer such conditions could explain, together with the SRT influence, the different microbial populations. The cake layer can be regarded as a more challenging place for microorganisms to thrive, due to the biomass compactness and consequently the lower diffusion of nutrients and oxygen through the layer, and also due to shear stress imposed by membrane scouring. Stress can imply a higher level of EPS production that in turn can affect the permeability of the membrane.

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The authors acknowledge Dr Ana Coelho and Dr Renata Soares from the Mass Spectrometry Laboratory, ITQB, and Fundação para a Ciência e Tecnologia (FCT) for the project PTDC/EBB-EBI/098862/2008 and grants SFRH/BD/40969/2007, SFRH/BPD/30800/2006, PEst-OE/EQB/LA0004/2011.

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CHAPTER 5

Role of extracellular polymeric substances in membrane fouling determined through membrane autopsy

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Silva AF was involved in all the experimental work presented in this chapter, except for the operation of the lab-scale membrane bioreactor and protein identification by MS. The reactor was operated by Antunes S and Silva C; protein preparation to protein preparation to be analyzed by MALDI-ToF/ToF and MASCOT search was performed by Vieira A.

CONTENTS

Abstract

Membrane bioreactors (MBRs) are an advanced technology with numerous advantages for wastewater treatment. MBRs' major obstacle for wider application is membrane fouling. Extracellular polymeric substances (EPS) are key foulants that deposit and adhere to the membrane surface and pores, forming the gel layer, and aid in the formation and consolidation of the cake layer. In this study we identified the major EPS foulants comprising the gel and cake layers of a MBR. The impact of the sludge retention time (SRT) on the composition of the EPS foulants was investigated by operating the system at SRTs of 60 and 20 days. At the end of each SRT, the cake layer was collected and the membrane modules were autopsied for the extraction of the gel layer, where several extraction methods were compared in terms of extraction efficiency of polysaccharides and proteins from internal and external fibres of the membrane module. Polysaccharides were the major EPS foulant in the gel layer, particularly in the internal fibres, and at the 20d-SRT period. Proteins and polysaccharides were present in similar concentrations in the cake layer at the end of the 60-d SRT period. However, a 20-d SRT resulted on a six fold increase of the protein concentration in this fouling layer. A significant part of the proteins identified from this second cake layer were enrolled in stress response. Others not conventionally related to stress response can be implicated in such function. The increase in EPS concentrations and the identification of a significant group of proteins related to stress response may indicate that MBRs operational conditions, particularly at lower SRT, may produce a stressful environment to the microorganisms, resulting in a higher accumulation of EPS at the membrane surface.

5.1. Introduction

Membrane bioreactors (MBRs) are increasingly applied in the treatment of municipal and industrial wastewater due to the advantages they offer over conventional wastewater treatment plants (Judd 2008). However, a major drawback to the use of MBRs technology is the progressive decrease in permeability, resulting in an increase in transmembranar pressure (TMP) to unsustainable values, majorly due to biofouling (Meng *et al*. 2009). Biofouling is formed by the deposition of cells and extracellular polymeric substances (EPS) on the membrane surface and inside its pores, which occurs in two steps. First, a slow increase in TMP occurs due to the formation of a gel layer on the membrane by means of deposition of soluble EPS, and, to a lower extent, of other colloidal material present in the wastewater. Secondly, the permeation flux drives biomass flocs and suspended solids towards the membrane, forming a cake layer, concomitant with a rapid increase of TMP (Wang and Wu 2009). Part of the biofouling can be removed through physical and chemical cleaning strategies, which are routinely applied to MBRs. However, the repeated cleaning cycles imply temporary interruption of the flux, and impose an additional stress on the membranes, reducing their life spam (Meng *et al.* 2009; Su *et al.* 2011; Malamis *et al.* 2011).

Sludge retention time (SRT) is one of the most important parameter affecting MBR fouling (Drews 2010). SRT impacts on the sludge ecophysiology, both the microbial community structure and the EPS proteins profile, as it was described in chapter 4 of this thesis. In literature SRT in general is described to lead to a higher fouling of the membrane when set for short periods of time, mainly due to high concentration of EPS (Malamis *et al*. 2011, Tian and Su 2012).

Few studies have addressed the EPS characterization of the fouling layer with no consensus on which EPS molecules (proteins or polysaccharides) has a great contribution to fouling (Germain *et al*. 2005; Ng and Ng 2008 ; Rosenberger *et al.* 2006 ; Lyko *et al.* 2007)). Recently, a few studies have been carried out to unveil the EPS protein and polysaccharide composition of fouled membranes in MBRs. (Al-Halbouni *et al.* 2008) analyzed polysaccharides from a full scale MBRs and verified that polysaccharides were all retained in the MBRs but only vestigial mannuronic acid was identified in the EPS extracted from the gel layer. *Miyoshi et al.* (2012) analyzed the EPS proteins from the gel layer but obtained very few results, identifying only two proteins from the outer membrane of *Pseudomonas* genus, OprF and OprD. Huang *et al*. (2012) found a correlation between certain fouling proteins with specific membrane material. These studies took a step forward in the direction of understanding fouling production and resistance to cleaning. However, to better understand the fouling process, it is important to deepen the level of characterization of EPS in both foulant layers, with equally important but different contributions on the membrane performance, at different operational conditions

In this work, we have characterized the cake layer attached to the membrane of a lab-scale MBR operated at two different SRTs (60 and 20 days). Moreover, the gel layer of internal and external fibers was also analyzed. In hollow fiber (HF) membrane modules with no internal aeration, the fibers are differently subjected to fouling according to their position in the bundle, since air scouring affect mostly the external fibers, and a thicker layer of cake is developed in between the inner fibers. To insure a correct extraction of proteins and polysaccharides, a set of five extraction methods from the internal and external HF fibers were compared. To the best of our knowledge this study is the first to

analyze EPS molecules responsible for membrane fouling on MBRs by characterizing the EPS composition of both fouling layers under different operational conditions.

5.2. Material and Methods

5.2.1. Membrane bioreactor

The activated sludge samples were collected from a MBR comprising a 13 L anoxic tank and a 17 L aerobic tank, with a submerged module of polysulphone hydrophilic HF membranes (Polymem, Tolouse, France), with an area of 0.1 m^2 and a pore diameter of 0.1 µm. Permeation was operated intermittently, with a relaxation period of 1 min every 10 min. The aeration flow (Qaer) was high (40 $Lmin^{-1}$) to promote membrane scouring. The MBR was first operated at a SRT of 60 days followed by a SRT of 20 days. The operational conditions are listed in Table 5.1 with average values throughout the two experimental periods. The feed was composed of domestic wastewater collected at the Mutela wastewater treatment plant (Almada, Portugal) supplemented with sodium acetate to maintain a constant food to microorganism ratio (F/M) throughout the operation.

Table 5.1: Operating parameters (average and standard deviation) for the lab-scale MBR during the two experimental periods

	60 d SRT	20 d SRT
HRT (h)	35 ± 13	44 ± 21
TMP (mbar)	297 ± 161	523 ± 207
F/M (h^{-1})	0.01 ± 0.00	0.01 ± 0.01
MLSS (gL^{-1})	5 ± 1	7 ± 1
VSS (gL^{-1})	4 ± 1	6 ± 1
pH	8.0 ± 0.5	8.0 ± 0.5
Temp (°C)	20 ± 2	20 ± 2

HRT: hydraulic retention time; TMP: transmembrane pressure; F/M: food to microorganisms ratio; MLSS: mixed liquor suspended solids; VSS: volatile suspended solids

5.2.2. Membrane autopsy and EPS extraction

The membranes were rinsed with saline buffer (2 mM Na_3PO_4, 4 mM $NaH_2PO_4 \bullet 12H_2O$, 9 mM NaCl, 1 mM KCl, pH 7) to remove the cake layer. The membrane modules were then autopsied as follow: four fibers from each module with exactly the same size were cut into small pieces, two from the outer side of the bundle (external fibers) and two from the inner part of the bundle (internal fibers). Five different methods to extract the EPS from the membranes were compared, based on EPS extraction methods from the suspended flocs described in the literature (Liu and Fang 2002; Comte *et al.* 2006 ; D'Abzac *et al.* 2010). All protocols started by a previous immersion of the small pieces of fibers in saline buffer (40 mL) and ended with the recovery of the EPS by centrifugation at 12,000xg for 20 min at 4 °C. The particularities of each protocol were: 1CP) In the control protocol the fibers were stirred in buffer at 600 rpm for 3 h at 4 °C; 2FN)

240 µL of 37% formaldehyde were added to the buffer containing the fibers, which was stirred at 600 rpm for 1 h at 4 °C. Next, 16 mL of NaOH (1 N) was added to the solution and stirred for 3 h at 4 °C. The supernatant recovered by centrifugation was dialyzed overnight at 4 °C to recover the EPS fraction; 3S) The fibers in buffer were sonicated in a ultrasound bath (300 ultrasonik, Ney) at 50% power for 4 cycles of 30 sec, alternated with 30 sec in ice; 4T) The fibers in buffer were stirred at 600 rpm for 3 h at 70 °C; 5CER) A cationic extraction resin (CER) was added to the fibers in buffer (Dowex® MARATHON®, 20–50 mesh, Sigma-Aldrich Co., St Louis, USA) in a proportion of 15 g per 60-80 cm^2, and stirred for 2 h at 4 °C.

Total protein content was determined by the Lowry method (Frolund *et al*. 1996), using bovine serum albumin (Merck KGaA, Germany) as standard. Total polysaccharide content was determined by the Dubois protocol (Dubois *et al.* 1956), and calibration was performed with glucose (Sigma Chemical Co, St Louis, USA). All protein and polysaccharide concentrations determined for the cake layer were normalized per cake biomass concentration, and for the gel layer, per area of membrane. Total extracellular DNA (eDNA) was determined with propidium iodide (D'Alvise *et al.* 2010) and calibration was performed with fish sperm DNA (Sigma-Aldrich Co. St. Louis, USA).

5.2.3. Protein identification

For protein identification, EPS extracted from cake and membrane autopsy samples were analyzed following the method described by (Silva et al. 2012). In summary, the samples were concentrated overnight and precipitated in parallel

with acetone 4× and trichloroacetic acid (TCA) 13%. The protein pellets were resuspended with NuPAGE® LDS sample buffer (4×) (Invitrogen, Carlsbad, CA, USA). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the NuPage® electrophoresis system instructions (Invitrogen, Carlsbad, CA, USA). The gel bands of interest were excised and in-gel-digested with trypsin as previously described (Santos *et al*. 2009). The procedure to identification of the proteins by mass spectrometry (MS) is detailed elsewhere (Silva *et al.* 2012).

5.2.4. Statistical analysis by box and whisker plots

Protein and polysaccharide concentration data obtained from the EPS extraction processes described above was illustrated graphically through box-and-whisker plots for assessing the distribution of a sample through its medians, means, quartiles, and minimum and maximum observations. The box's upper boundary represents the upper quartile (75^{th} percentile) of the data and the lower boundary the lower quartile (25^{th} percentile). The length of the box represents the interquartile range (IQR) and the median is demonstrated by a straight line drawn inside the box. Whiskers in a box plot are drawn from the upper and lower edges of the box representing the largest and smallest observations, respectively. The small circle symbols denote outliers, i.e. values above or below the threshold defined as 1.5-fold of IQR.

5.3. Results

5.3.1. Concentration of proteins and polysaccharides in cake and gel fouling layers

The EPS extracted from the cake layer recovered from the membrane module at the end of each studied period presented distinct protein and polysaccharide contents (Table 5.2). EPS proteins and polysaccharides in the cake layer were present in similar concentrations when the bioreactor was operated at SRT of 60 days. When the SRT was reduced to 20 days, the protein content increased to a value 5-fold higher (Table 5.2).

Table 5.2: Quantification values (average and standard deviation) determined for the cake layer EPS proteins and polysaccharides at the end of each SRT

SRT	Proteins (mg $gVSS^{-1}$)	Polysaccharides (mg $gVSS^{-1}$)
60	8.89 ± 0.03	7.08 ± 0.05
20	43.13 ± 1.54	9.41 ± 6.86

The EPS extracted from the gel layer presented different outcomes in respect to proteins and polysaccharides for the various methods studied in this work (Figure 5.1). It should be noted that the amount of DNA determined after each extraction protocol was negligible, indicating that the polysaccharides and proteins determined did not result from cell lysis in any of the methods. Therefore, the most successful extraction protocols were those retrieving the highest concentration of EPS molecules.

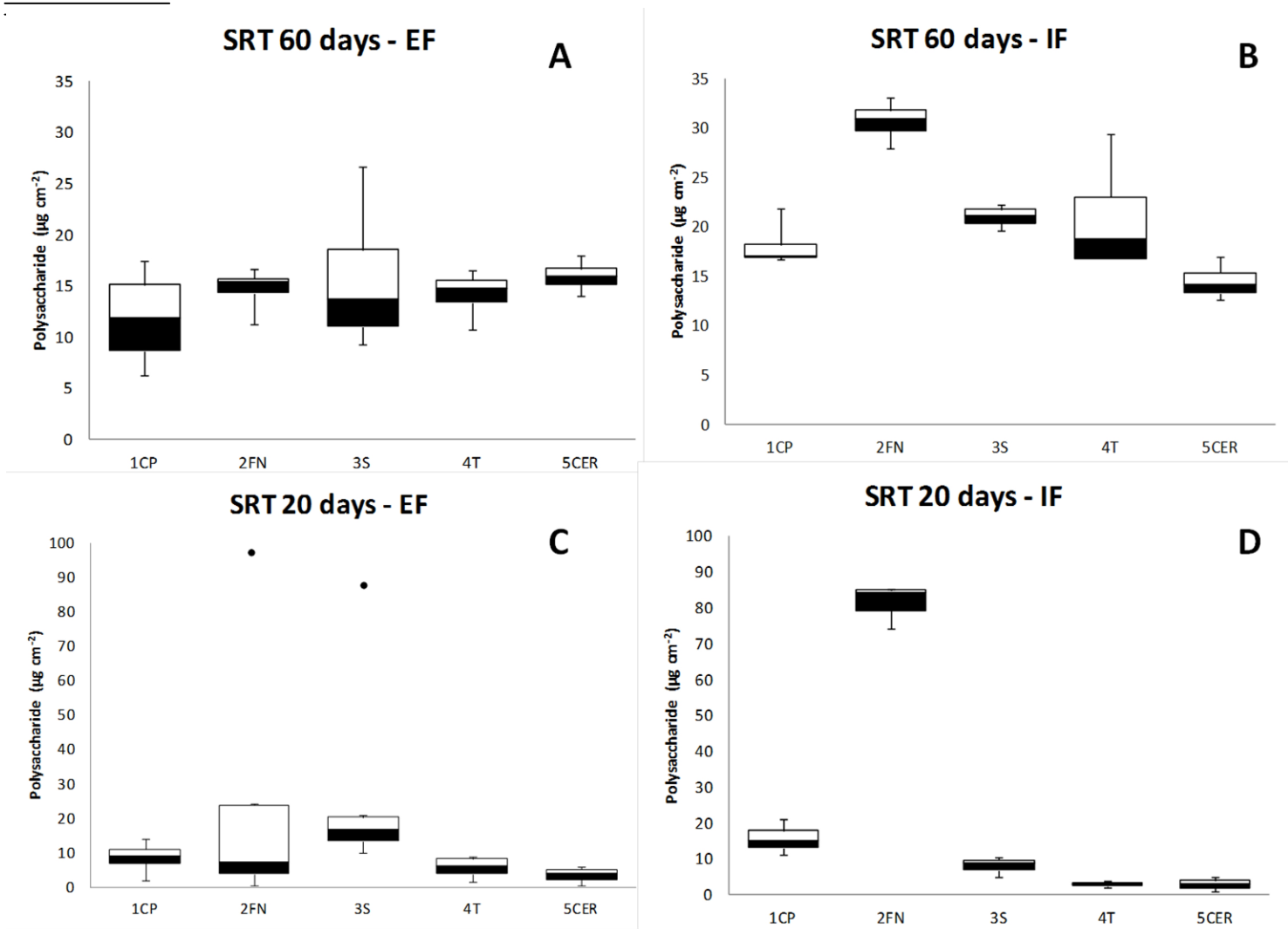
Membrane autopsy carried out after the 60 d SRT period, for the external fibers, showed no significant difference between the different extraction protocols tested in terms of quantity of polysaccharides retrieved. However, the CER protocol seemed to give more consistent results, with less dispersion between replicates (Figure 5.1A), with a median of 15.9 ± 0.7 μg cm^{-2}. However, for the internal fibers, the best extraction method for EPS polysaccharides was formaldehyde/NaOH with a median of 30.9 ± 1.1 μg cm^{-2}, where the other protocols could only retrieve less than 2/3 of that value (Figure 5.1B). Thus, comparing the best extraction methods for the two types of fibers, the EPS comprising the gel layer on the internal fibers had twice the polysaccharide content of the external fibers.

For the 20 d SRT period, the EPS polysaccharides in the gel layer were best extracted from external fibers with the sonication protocol (median 17.0 ± 3.5 μg cm^{-2}) (Figure 5.1C). For the internal fibers the formaldehyde/NaOH method was clearly superior (median 84.0 ± 5.1 μg cm^{-2}) to the remaining methods (Figure 5.1D). Comparing the best extraction methods, the internal fibers had five times higher polysaccharide content than the external fibers.

Comparing the polysaccharides concentrations from each type of fibers under the different SRT periods, the polysaccharide content at the external fibers surface was maintained during both SRT periods. But, for the internal fibers, in the shorter SRT, the polysaccharide concentration increased 2.5 fold.

EPS protein concentration in the gel layer was very low comparing to the polysaccharides content (Figure 5.1E and F). Proteins were only detected by one of the extraction methods for each of the SRT conditions. The best protocol was different for SRT of 60 d (CER protocol) (Figure 5.1E) and 20 d (temperature

protocol) (Figure 5.1F), but for each operational conditions, the results were consistent for inner and external fibers. Although protein concentration in each SRT was low, there was a slight increase in the 20 d SRT gel layer, similarly to what was observed for the cake layer, but not so pronounced.


SRT 60 days - EF
A
Polysaccharide (µg cm-2)
0
5
10
15
20
25
30
35
1CP
2FN
3S
4T
5CER
SRT 60 days - IF
B
Polysaccharide (µg cm-2)
0
5
10
15
20
25
30
35
1CP
2FN
3S
4T
5CER
SRT 20 days - EF
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Polysaccharide (µg cm-2)
0
10
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1CP
2FN
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5CER
SRT 20 days - IF
D
Polysaccharide (µg cm-2)
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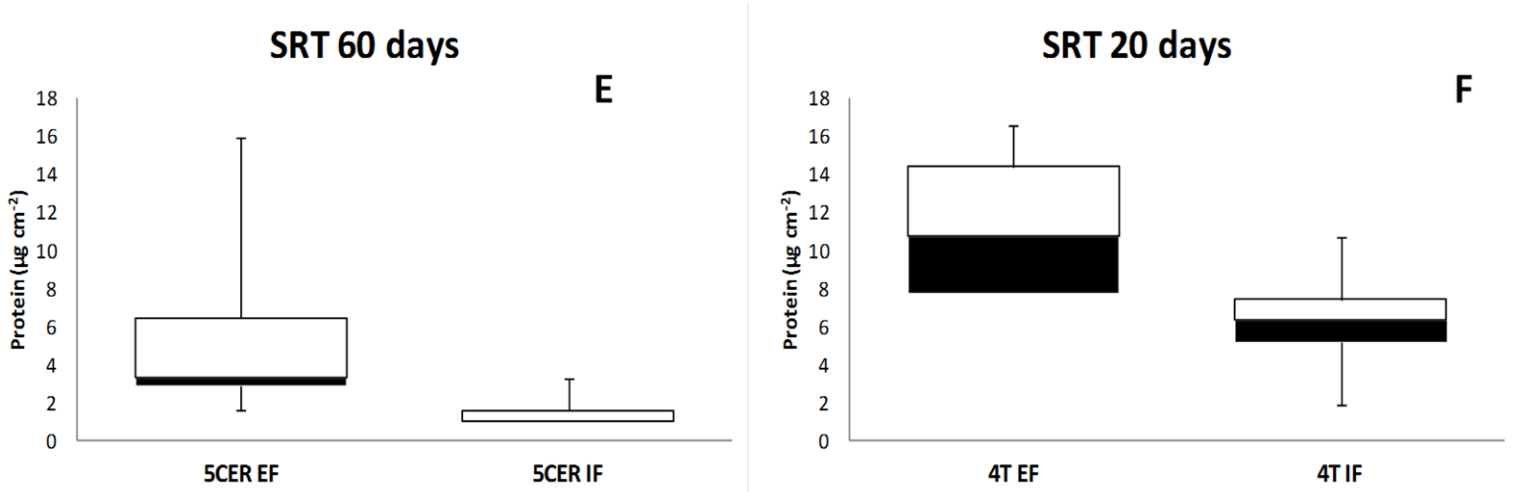

Figure 5.1: Box and whiskers plot of the polysaccharide (A-D) and protein (E,F) quantification results of the EPS extraction through membrane autopsy (gel layer) after MBR operation at SRT of 60 d and 20 d. CP-control; FN-formaldehyde + NaOH; S-sonication; T-temperature; CER-cation exchange resin; EF: external fibers, IF: internal fibers

5.3.2. Protein identification

EPS proteins from the gel layer and from the cake were concentrated and precipitated, followed by separation by SDS-PAGE, as per Silva *et al*. (2012).

For both operation periods (60 d and 20 d SRT), the gel layer proteins resulted in a single very faint band in the SDS gel (data not shown). Nevertheless, the few tenuous bands were cut and tripsin-digested for analysis by MS, although no results were obtained by MALDI-ToF/ToF.

The cake layer EPS proteins from the 60 d SRT period were few and represented by faint bands in gel (Figure 5.2A), regardless the protein precipitation method applied (TCA or acetone), which was expected taking into account the low protein concentration (Table 5.2). Nevertheless, the few visible bands were cut and prepared for MS analysis.

For the cake layer EPS proteins of the shorter SRT it was possible to visualize several bands in gel, which was in agreement with the total protein concentration of this sample (Figure 5.2B). Bands were cut off from the acetone precipitation protocol and prepared for MS analysis.

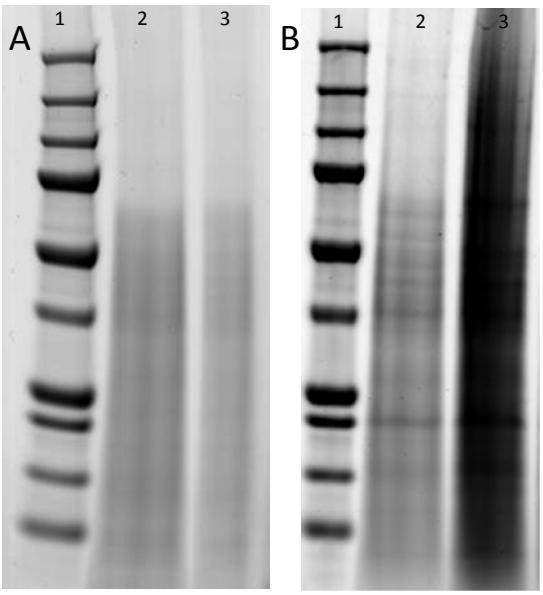

Figure 5.2: SDS-PAGE of cake layer EPS proteins precipitated with acetone (lanes 2) and with TCA (lanes 3). A) EPS proteins from the cake layer extracted at the end of the 60d SRT period; B) EPS proteins from the cake layer extracted at the end of the 20d SRT period

Due to the low protein concentration for the EPS of the 60 d SRT cake it was not possible to obtain any protein identification by MS analysis. From the cake layer EPS extracted at the end of the 20 d SRT fourteen proteins were identified belonging to 11 protein species (Table 5.3), with molecular weights below the pore size of the membrane (300 kDa).

Table 5.3: EPS proteins from cake layer at the end of the operation period with SRT of 20 days. Identification was obtained by MS analysis from SDS-PAGE protein profile bands and database search at Protein Knowledgebase UniProtKB/Swiss-Prot database

Protein species	No.
Hypothetical protein HMPREF1135_01892	1
Hypothetical protein HMPREF0491_01465	1
Bacterial stress protein	1
Tellurium resistance protein TerD	1
Acetyl-coenzyme A synthetase	2
60 kDa chaperonin (GroEL gene)	1
30S ribosomal protein S4	1
Glutamine synthetase	1
Cytadherence high molecular weight protein 2	1
Isocitrate lyase	2
AceA protein	1
ABC transporter substrate-binding protein	1

Approximately half of the proteins identified are located in the cell membrane (Figure 5.3A). A number of them are enzymes involved in biosynthetic processes and another big group is produced as a response to stress (Figure 5.3B). These two biological roles are known to be common functions among proteins in conventional biofilms (Flemming and Wingender 2010).

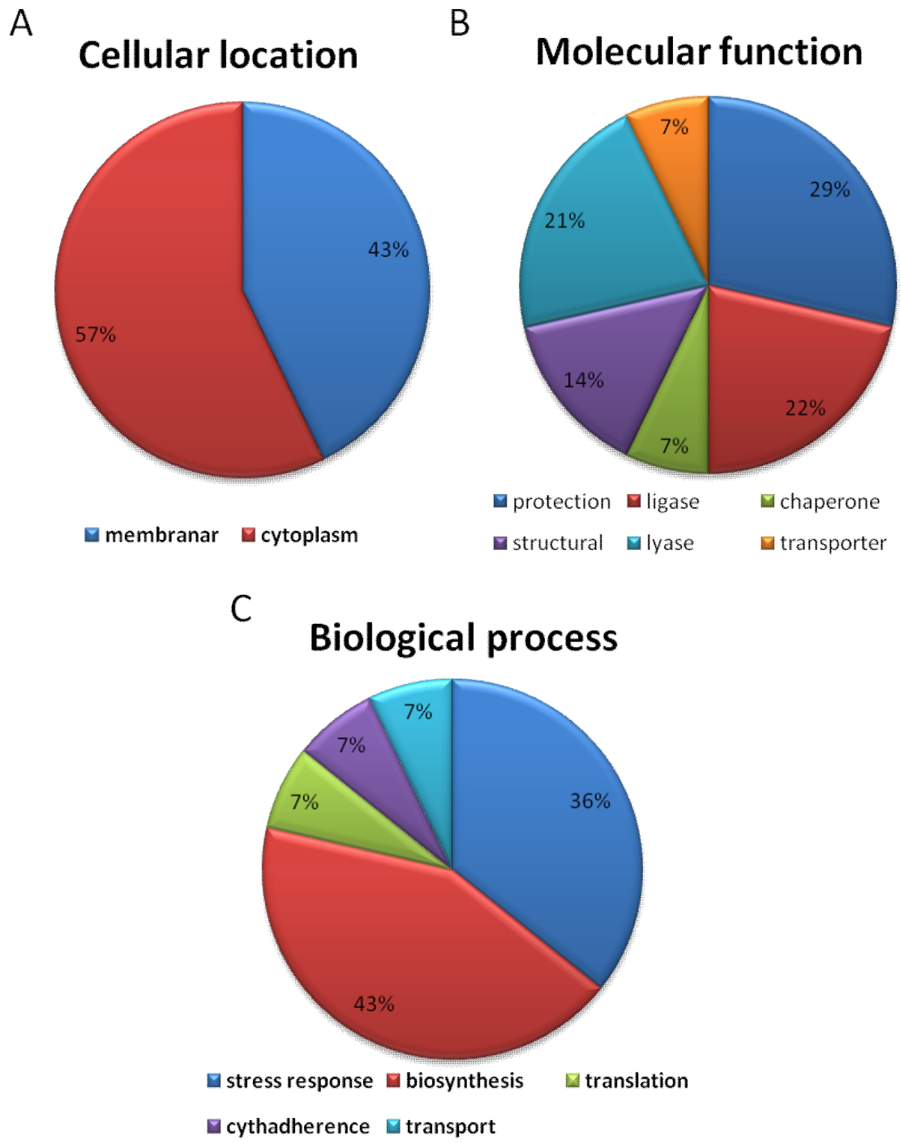

Figure 5.3: EPS Protein results organized by cellular origin (A), metabolism function (B) and by biologic process in which they are involved (C), according to Protein Knowledgebase UniProtKB/Swiss-Prot database.

5.4. Discussion

The biofouling in MBRs emerge by the deposition of biopolymers and cells at the membrane surface. Two layers are formed at the surface of the membrane during the filtration process, the gel layer and the cake layer, both with extreme importance on the permeability of the membrane. In this work, the impact of operational conditions on fouling was investigated at a molecular level, through a comprehensive analysis of the EPS composing the two fouling layers when the reactor was operated at different sludge retention times.

From the analysis of the EPS composition of the gel layer, proteins were detected in low abundance and only by one of the methods at each operation period, independently of the localization of the fiber: the CER for the 60 d SRT period and the temperature method for the 20 d SRT period. This difference in results for the biomass enriched in the two operation periods probably reflects the type of binding forces maintaining the proteins in the EPS matrix, which could be due to the presence of different proteins, different surrounding molecules or environmental conditions. Although always within low values, proteins concentration at the end of 20 d SRT period was superior to the one from the 60 d SRT period, which could be a response to the new environment that the microorganism were subjected to at the shorter SRT, namely a higher MLSS and TMP. This results contradicts previous studies where proteins were described as major foulant molecules in MBR systems for wastewater treatment (Miyoshi *et al*. 2012; Huang *et al*. 2012). A hypothesis to explain the overall low abundance of proteins found in our study is that proteins were mostly expressed in a particular stage of the biofilm evolution, not taking place at the time of the autopsy. This kind of differential expression of proteins was observed for example in the biofilm

of *Acidithiobacillus thiooxidans*, in which proteins were significantly expressed 46 h after the colonization by the bacteria (Garcia-Meza *et al*. 2013)). Moreover, Ou *et al*. (2010) described that polysaccharides adhere firstly to the membrane and that protein concentration increased with time in a submerged MBR. In our case, the fouling layers were analyzed after three sludge retention times and in steady state conditions, which might have been long enough time for proteins to accumulate in the cake layer and disappear from the gel layer.

Polysaccharides had a preponderant role in the gel layer: they were abundant both in internal and external fibers, as detected by all the methods tested at the two different operational periods. These results are in agreement with publications in which polysaccharides were detected at the membrane surface and obstructing the membrane by adhering inside the pores. Moreover, polysaccharides are responsible by the structure of the gel layer, and a driving force for the increase in TMP (Drews 2010); (Wang and Wu 2009) (Chu and Li 2005, Ng and Ng 2008). In this study, independently of the SRT imposed, the internal fibers had gel layers with higher polysaccharide content than the external fibres. In the design of a HF membrane module the external fibers are subjected to a higher shear stress by the aeration flow, which in our case was set to promote the scouring of the membrane. The internal fibers are more sheltered from this cleaning procedure and the accumulation of biomass and growth of the biofilm proceeded with more success. Supporting this statement was the observation of a dense cake layer in the internal part of the module, and a much finer cake being formed on the external fibers (data not shown). For the proteins, the scenario was the opposite: although with small differences, the external fibers had always higher protein content. A hypothesis to explain the data is that the protein content of EPS increases in stressful situations, as high shear stress.

In the gel layer the polysaccharide to protein ratio was always higher than 1, considering the best extraction method for both molecules. Most of the polysaccharides are described as longer molecules than proteins with a molecular weight (MW) between 500 kDa to 2000 kDa (Flemming and Wingender 2010)). One can speculate that polysaccharides are being retained at the surface and pores of the membrane but proteins could either have passed through the membrane with the permeate, or were trapped in the cake layer. Supporting the former hypothesis was the presence of proteins in the permeate at the moment membrane was autopsied for the 60 d SRT period (1.93 mg g^{-1}VSS – data normalized with the VSS from the mixed liquor). The latter hypothesis is well supported by the quantification results obtained for the cake layer of the 20 d SRT and approximately no protein detected in the permeate around the date of autopsy (0.00 ± 0.00 mg g^{-1}VSS - data normalized with the VSS from the mixed liquor). Indeed, in a review of the implication of cake sludge on membrane fouling (Wang and Wu 2009), the cake is pointed out, until a certain thickness, as a screen of foulants species of small size, improving the membrane filterability.

The EPS composition of the two cake layers layers (at the end of the 60d and 20d SRT periods) was different from the corresponding gel layers. In this case, proteins were the preponderant EPS molecule comprising the cake layers. These results are in agreement with studies in which proteins were dominant in the cake layer (Hu *et al*. 2013), and that throughout the thickness of the cake, the concentration of proteins decreases when in close contact to the membrane (Gao *et al.* 2011). In fact, in our study proteins were not observed in the gel layer. Although in the 60 d SRT the difference between proteins and polysaccharides was insignificant, in the following SRT condition (20 d) the protein concentration increased considerably in respect to the polysaccharides, which did not vary

significantly between SRTs. SRT is an operational parameter known to indirectly affect the fouling of the membrane through the impact on sludge characteristics (Drews 2010). A low SRT normally leads to a higher membrane fouling due to the increase on EPS concentration of the mixed liquor (Van den Broeck *et al*. 2012 ; Su *et al.* 2011). In agreement with such reports, in this study we also observed an impact in the cake layer EPS molecules concentration with the change for a lower SRT. This results can be related to the fact that a lower SRT implies younger and more active microorganisms in the suspended biomass, and consequently in the cake.

Proteins were only identified for the cake layer from the 20 d SRT period due to the low concentration of proteins in the 60 d SRT cake, reflected on the faint bands on the gel. Analyzing the protein grouping by the molecular function and biological processes (Figure 5.3), a very significant part of the proteins are recognized as stress related, as the chaperone GroEL, the hypothetical proteins, the stress protein and the tellurium resistance protein TerD, according to UniProtKB/Swiss-Prot and NCBI protein databases. Other proteins, conventionally related with different functions, can also be implicated in stress response. ABC transporters, which are known to be involved in the secretion of the polysaccharides and extracellular enzymes in biofilms (Wingender *et al.* 1999), have also been involved in oxidative stress tolerance for *Streptococcus mutans*, a bacteria responsible for dental biofilms (Seaton *et al*. 2011). Given the discussed identification results and the fact that proteins were highly expressed in the cake layer from the 20 d SRT period in comparison to the 60 d SRT period, it seems that the conditions experienced at the 20 d SRT are stressful to the microorganisms present in the cake layer. The identification of adherence proteins, involved in the biofilm formation, and enzymes such as lyases, which are polymers' degraders,

were in agreement with commonly described proteins found in biofilms (Wingender *et al*. 1999). The proteins normally known as intracellular most probably are release by natural cell decay or by cell leakage, staying trapped in the matrix in close contact to the cells. Alternatively, they may also be excreted with a particular purpose. For example, glutamine synthetase is known to be involved in the intracellular metabolism of nitrogen, but, in *Mycobacterium tuberculosis* it was suggested to be intentionally exported to the extracellular medium (Harth *et al.* 1994).

The results presented in this work represent a step forward in the fouling characterization of MBRs. The two main EPS molecules, proteins and polysaccharides, were found to be implicated in the fouling of the membrane but with different predominance in separate layers: the polysaccharides are an important foulant agent in the gel layer whereas the proteins are more relevant in the cake layer. From the protein identification data this result may be related with a response to stressful conditions. Also, a comprehensive assessment of the best methods for a successful extraction of EPS molecules was presented, which in the future could help in the comparison of EPS proteins and polysaccharide concentrations between different studies.

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CHAPTER 6

Discussion and Future Work

The biological treatment of wastewater relies on the capacity of the microorganisms to remove the organic matter, nutrients and pollutants from the wastewater. The microbial community thrives in wastewater treatment plants (WWTP) in an aggregative form, which is possible due to the excretion of extracellular polymeric substances (EPS), which form a matrix that capture the different microorganisms in flocs, granules or biofilms. Other functions beyond the aggregation of the biomass are important for wastewater treatment, such as trapping the wastewater molecules making it more readily available to the microorganisms.

Membrane bioreactors (MBRs) are an advance technology that completely separates solids from the water through the presence of a membrane. MBRs combine the biological treatment with a filtration step which results in a high quality effluent and WWTPs with smaller dimensions. These are extremely important advantages in view of the limited available footprint for the construction of new treatment systems and the more stringent regulations imposed for environmental discharges. Also, the treated effluent can be directly reused for non-potable applications, without the need of further disinfection procedures.

In such systems we have a loop of influences between the membrane and the microorganisms. The conditions offered by the MBRs to the microbial populations are different from the ones of a conventional treatment system: the typical MBRs operational conditions such as the possibility to operated the reactor at long sludge retention time (SRT), can promote proliferation of different microorganisms, with an improvement on the biological nutrient removal; but MBRs can also impose potentially stressful conditions such as spatial constrictions

due to a higher biomass concentration and to shear stress that comes from high aeration flow rates imposed for scouring of the membrane. The microbial population replies to the specific conditions inside a MBR by presenting a dynamic population and excreting more EPS and/or different EPS molecules. The microbial cells and the EPS produced impact in the MBRs performance by causing the fouling of the membrane and compromising the membrane filterability.

In this study we addressed the potential of MBRs to enhance the biological nutrient removal as well as the response of the microbial populations to the presence of a membrane and to the specific operational conditions of a MBR.

The first part of this thesis investigated the capacity of the microorganisms involved in biological nutrient removal to thrive in MBRs, with particular emphasis on the populations related to phosphorus removal. The activated sludge of eight European MBRs treating municipal wastewater was screened for these microorganisms by fluorescence *in situ* hybridization (FISH) using a comprehensive set of probes. Results showed that, among the general population, the communities were dominated by *Betaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* classes. These results were in agreement with former studies concerning the microbial population of conventional activated sludge as well as MBRs (Luxmy *et al.* 2000; Witzig *et al.* 2002; Wagner *et al* 2002; Sofia *et al.* 2004). Among the nutrient removal populations, the ammonia oxidizing bacteria (AOB) were very low represented or absent in the MBRs. For the nitrite oxidizing bacteria (NOB), only *Nitrospira* was detected. Recent studies applying next generation sequencing to MBRs microbial populations detected both AOB and NOB communities, which were composed mainly by *Nitrosospira* and *Nitrosomonas* for AOB and *Nitrosospira* for NOB (Ma *et al.* 2013; Saunders *et al.*

2013). *Nitrobacter* was not found in any of these studies, not even in a survey of 5 MBRs designed for nitrification and denitrification, and it was postulated that *Nitrospira* need a lower nitrite concentration to grow and outcompete *Nitrobacter*. In what respects phosphorus removal, the polyphosphate accumulating organisms (PAOs) and their direct competitors for carbon source (glycogen accumulating organisms, GAOs) were targeted by a comprehensive set of probes. Other microorganisms regarded as putative PAOs and GAOs were also searched for in the MBRs samples. Of the eight MBRs, the higher phosphorus removal was achieved by the two designed for enhanced biological phosphorus removal (EBPR). However, the highest abundance and diversity was verified in one non-EBPR MBR, which had the highest phosphorus removal rate among the plants without an anaerobic tank. In fact, *Accumulibacter*, the model PAOs microorganism, was present in very low abundance in one of the EPBR-MBRs and the phosphorus removal may have been performed by the putative PAOs present, like *Actinobacteria*. Currently the *Tetrasphaera*-related *Actinobacteria* is recognized to have an active role in the removal of phosphorus in WWTPs (Gebremariam *et al*. 2011; Mielczarek *et al*. 2013). GAOs do not seem to find favorable conditions to proliferate, even in MBRs with low phosphorus removal. Probably the existence of a defined anaerobic zone is necessary for them to gain competitive advantage over the other heterotrophic bacteria. Recent studies combining FISH with high throughput sequencing techniques confirmed these results, in which PAOs were shown to proliferate over GAOs in MBRs, mainly in the ones not designed for EBPR (Saunders *et al*. 2013). MBRs offer conditions to PAOs to thrive although their activity as phosphorus removers is dependant of the operational conditions. In MBRs, PAOs may have found conditions beyond the presence of a defined anaerobic zone. MBRs are operated at low food to

microorganism (F/M) ratio, as well as high biomass conditions that can create anaerobic micro-niches. This type of confined environment may have occurred in the non-EBPR MBR with the highest phosphorus removal yield and PAOs abundance, which also had the highest mixed liquor suspended solids (MLSS) among all MBRs. Also, PAOs may have a selective advantage in these conditions of limited ATP supply with their polyphosphate granules serving as reservoirs of energy. This type of advantage was observed at the laboratory scale in a constant aerobic sequence batch reactor where the biomass was cycled between feast and famine stages, resulted in a selective enrichment of microorganisms, PAOs, capable of alternately accumulate phosphorus and poly-β-hydroxyalkanoates (PHA) (Ahn *et al.* 2007).

This work was pioneer in extensively characterizing the PAOs community in MBRs. It demonstrated the capacity of MBRs to promote the proliferation of important microbial populations for biological nutrient removal and to offer favorable conditions to perform such activity. In conventional wastewater systems the stability of EBPR is easily disturbed and GAOs proliferation is one of the most common causes. In the future it would be interesting to define the operational conditions that lead to the proliferation of PAOs over GAOs in MBRs and to promote further the activity of PAOs thriving in these systems.

EPS forms the matrix that maintains the cells together for the microorganisms to live in the form of biofilms, flocs and granules and it is very important regarding the performance of the MBRs, both in the treatment of the wastewater and in the fouling of the membrane. In this thesis a new strategy was designed that allowed the identification of the proteins composing the EPS. In the literature several attempts were described to identify such macromolecules but

had scarce outcomes. Protein concentration and co-presence of other EPS molecules seemed to be the major issues. Also the resolution of the proteins bands in gel had to be improved. This work tested several procedures and overcame these obstacles, defining a step by step strategy that enables the identification of a high number of EPS proteins. At the end of this study a successful workflow was designed with the following steps: Proteins are concentrated by the method of coating dialysis tubes with polyacrylate-polyalcohol (PP) absorbent gel. Next, proteins are further concentrated and precipitated from the remaining EPS molecules by acetone (for soluble EPS) and TCA (for soluble and bound EPS) precipitation protocols. Proteins are applied in a gradient SDS-PAGE gel to achieve a good band resolution and the trypsin digested peptides are run in a MALDI ToF/ToF mass spectrometer. To the best of our knowledge, it was showed for the first time the complexity of the metaproteome of activated sludge in MBRs. After this a similar strategy has been applied to study the EPS proteins in MBRs with fairly good results in gel (Miyoshi *et al.* 2012). In the future, this strategy could be upgraded using two dimensional gel electrophoresis to permit the concomitant identification and quantification of each protein. Also the inclusion of an additional purification step could be considered to extract the proteins from the remaining EPS molecules that hamper the analysis of samples with a high concentration of such components, like humic acids.

As referred in the beginning of this chapter, the microbial population and the EPS are fundamental elements in biological wastewater treatment systems. In conventional wastewater treatment plants these mixed communities and matrix are fairly uncharacterized, but this black box is even darker for the MBRs' activated sludge. In this thesis, the structure of the microbial populations of the suspended flocs and the cake layer formed on a lab-scale MBRs membrane were

unraveled by high throughput sequencing. The impact of the operational conditions was studied and the dynamics of the population was verified throughout a first SRT period of 60 days, followed by a period of 20 days. The extracellular metaproteome of the suspended flocs communities was also investigated by the strategy described in chapter 3. It was observed that the microbial community between the suspended flocs and the cake layer was not significantly different in the longer SRT period. But the shorter SRT highly impacted on the population structure of the cake layer. A clear shift occurred in the population structure during the adaptation period, ending with a different community profile among the suspended flocs and the cake layer. Within the bacterial population from the mixed liquor, *Gammaproteobacteria* and *Alphaproteobacteria* were the most prevalent classes. The cake layer shared the same dominant groups during the 60d SRT but in the 20d period the predominant class was *Actinobacteria*. The results for the suspended flocs are in agreement with recent studies based on high throughput sequencing of the metagenome of MBRs populations treating municipal wastewaters (Lim *et al*. 2012; Ma *et al*. 2013; Kim *et al*. 2013) but it disagrees with former studies based on FISH and DNA fingerprints techniques, from which *Betaproteobacteria* were always the most relevant class in the microbial community of the mixed liquor (Gao *et al.* 2013; Luxmy *et al.* 2000; Witzig *et al.* 2002; Sofia *et al.* 2004). The sensibility and coverage capacity of these new techniques is enormous and may be the reason for such differences between former and current studies. In the future the difference between the population of the mixed liquor and cake layer shown in this study could be explored regarding the identification of particular microorganisms that have key roles in biofouling. The metaproteome from the suspended flocs throughout the two SRT periods showed a high diversity. A

significant part of the proteins were described in the Uniprot database as cytoplasmatic. In the future, the EPS proteins could be tested for enzymatic activity and analyzed for cellular location prediction to clarify if they are intracellular or extracellular elements of the EPS. By principal component analysis (PCA), it was verified that the soluble and bound EPS behave as distinct fractions of the EPS, and that SRT is one of the operational conditions affecting the protein profile. A group of 14 proteins are only observed in the soluble EPS and mainly during the longer SRT period. This exclusivity had already been observed in chapter 3, in which, for example, porins were only detected in the soluble EPS fraction. Most of the identified proteins are directly related or have the potential to be related to protection/stress response, and were present in comparable numbers in the two SRT periods. Therefore SRT does not seem to be the main parameter responsible for the stress to which the microorganisms are apparently exposed to in MBRs. Stress can imply a higher level of EPS production that in turn can affect the permeability of the membrane. In the future the influence of other MBRs specific operational conditions and parameters on the ecophysiology of the microbial community could be studied to confirm if the biomass is in fact in stressful conditions and, if so, how the operational conditions can be manipulated to overcome these situations.

Finally, in this thesis a characterization of the EPS foulants was performed. Fouling occurs due to the establishment of two layers at the surface of the membrane. The gel layer origin is the deposition and adherence of soluble EPS molecules at the surface and inside the pores of the membrane. This deposition facilitates the colonization of the membrane by the microorganisms and formation of the biofilm. The cake layer occurs due to the transport and compression of sludge flocs against the membrane due to the suction force of the

permeate flux, and bound EPS contribute to the consolidation and maturation of this biofilm. In this study gel and cake layers were analyzed and different methods were applied to best retrieve the EPS foulants. There is no gold standard to extract EPS, which would made comparisons between researches a more feasible task, but the efficiency and sensibility of different methods are documented for the mixed liquor EPS (Liu and Fang 2002; Comte *et al.* 2006; D'Abzac *et al.* 2010). Based on such studies, five different methods were selected to ensure a comprehensive extraction of the EPS foulants from the gel layer. For the cake layer, the cationic extraction resin method was chosen, based on previous studies which demonstrated its suitability for sludge samples. It was taken into account that in a hollow fiber membrane module, the fibers differently located in the bundle are subjected to different scouring pressures. Additionally to this method optimization, the impact of the operational conditions was investigated by changing the SRT from 60 d to 20 d. This study demonstrated that proteins and polysaccharides have different contributions to membrane fouling. Polysaccharides were the major EPS molecules composing the gel layer from the two SRT periods and regardless the location of the fiber, with particularly high accumulation in the internal fibers. Proteins were found to be a important EPS molecule in the composition of the cake layer. Also a shorter SRT seems to be related to the increase of the main EPS foulants in both layers and an average higher transmembrane pressure was registered during this period. The proteins identified from the cake layer from the SRT period of 20 days revealed a high percentage (36%) of stress related proteins. This result is in agreement with the results regarding the EPS proteins identified from the mixed liquor throughout the same operational period, described in chapter 4, and reinforce the hypothesis of the biomass being exposed to a stressful environment. To the best of our

knowledge this is the first study to characterize the composition of the two fouling layers, with identification of part of the EPS intervenient, the proteins. The identification of the sugar composition of the polysaccharides fraction will be a great contribution to this work, completing the characterization of the major EPS foulants. In the future different operational conditions could be tested to assess if stressful conditions are correlated with a fouling trend. These results could be used to monitor the state of the biomass and to correlate fouling of the membrane with the type, and quantity, of proteins being excreted. This information would improve our understanding of the conditions that increase fouling, which could then be used to define operational conditions that should be reduced or avoided as a fouling minimization measure. Also biochemical characterization of the proteins and polysaccharides, like their hydrophobicity/hydrophilicity and adsorption potential, will be important to assess the type of interactions between EPS molecules and the membrane.

The potential of MBRs in view of the current water demand and scarcity worldwide is enormous. In the last two decades the MBR technology evolved greatly and currently is an established technology for municipal wastewater treatment. Two types of studies contributed to this evolution: the research with a practical point of view and the fundamental studies. This thesis fits in the latter group, focused on the microbiological characteristics of MBRs. The characterization of the microbial community and of the EPS produced by such populations in MBRs is a valuable tool to understand the functional potential of the system, as different bacterial populations have different functional characteristics that impact in the wastewater treatment. On the other hand, the microbial population structure is shaped by the bioreactor inherent characteristics and the operational conditions imposed. This thesis, while basically fundamental,

establishes pioneer correlations between operation/microbiology/performance in MBRs.

The identification of the microorganisms and proteins involved in the fouling of the membrane, the last major drawback for widespread application of MBRs, is an important information to the development of specific strategies to control or suppress their growth and expression, respectively. From results presented in this thesis, polysaccharides were found to be the most relevant foulants in gel layer. This suggests that chemical cleaning procedures should be optimized for this type of molecules, while the best membrane materials seem to be those minimizing adhesion of polysaccharides, known to possess a general hydrophilic nature. Another important practical aspect that could be deduced from the results of this thesis is the fact that many of the proteins found in the cake layer are stress-related. One of the possible stress factors is the intense aeration flow imposed in MBRs for membrane scouring. While this praxis is intended to reduce fouling, it is possible that it has the reverse effect by enhance the excretion of extracellular stress-response proteins. Also, the microbial composition of the MBRs was thoroughly analyzed using molecular tools: FISH for phosphorus removal related organisms and high throughput sequencing for the overall microbial structure. These studies showed for the first time that MBRs have a high potential to induce the growth of phosphorus removing organisms, and that the microbial populations at the foulant layer in situations of lower permeability are a specialized microbial group. Sludge retention time could be regarded as an adequate parameter to control the microbial population in the foulant layer and consequently the type of EPS produced.

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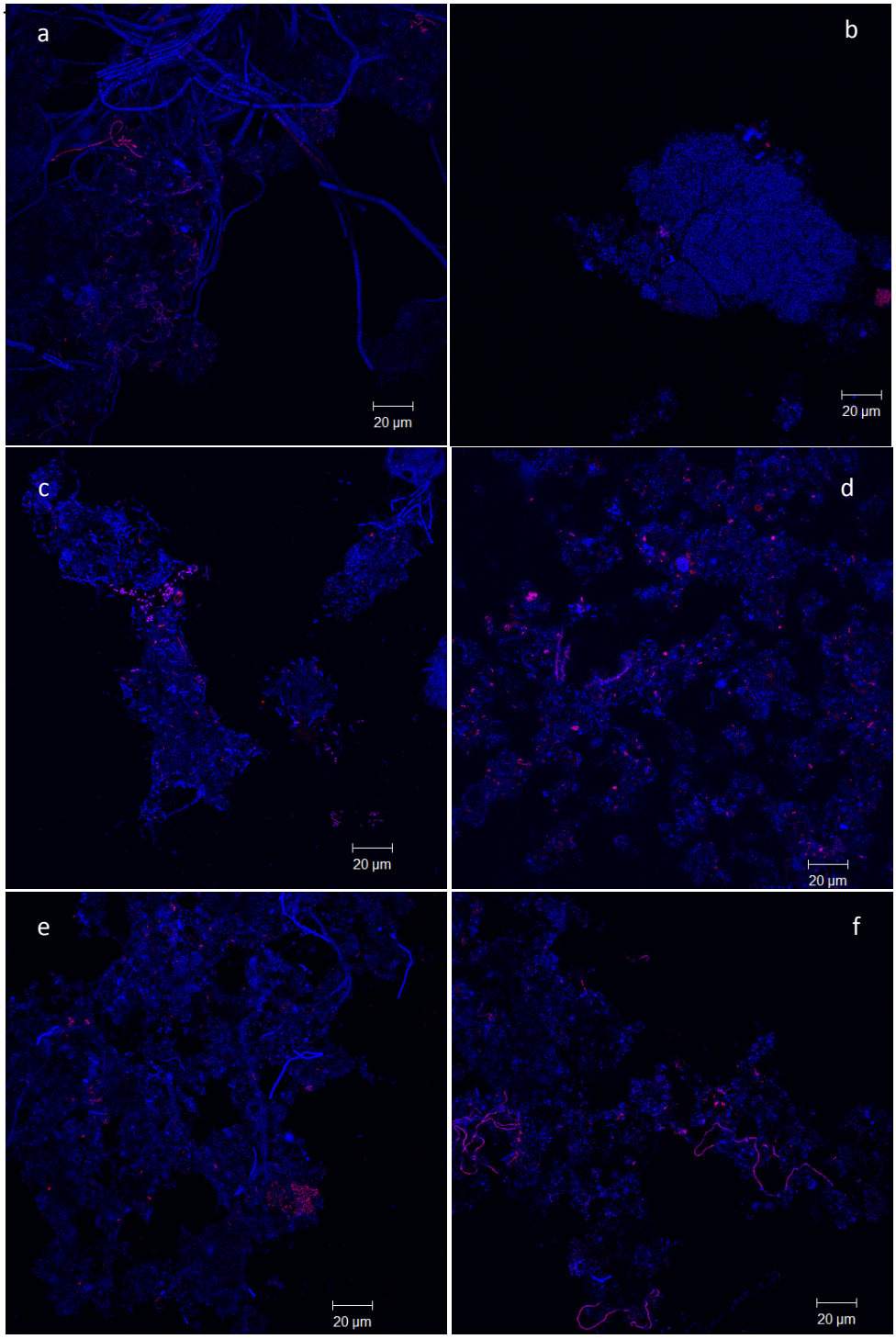
SUPPLEMENTARY MATERIAL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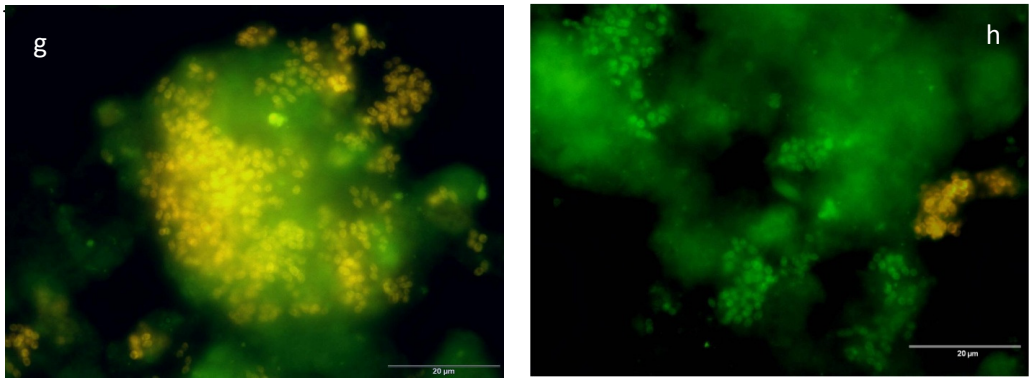

Figure S2.1: FISH micrographs (a to f CLSM, EUBmix in blue and specific probe in magenta; g and h epifluorescence, EUBmix in green and specific probe in yellow) from MBRs biomass samples. (a) EAWAG, specific probes Actino221 and Actino658, (b) Margaretenhohe, specific probes Actino221 and Actino658, (c) NTNU, specific probes Actino221 and Actino658, (d) Trento, specific probe Bet135, (e) Schilde, specific probe Bet65, (f) Schilde, specific probes Actino221 and Actino658, (g) Schilde specific probe Acc-I-444, (h) Schilde specific probe Acc-II-444

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